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INTERFERONS AND DERMAL FIBROTIC DISORDERS: NITRIC OXIDE PRODUCTION AND TRANSFORMING GROWTH FACTOR-β1 GENE EXPRESSION BY NORMAL AND HYPERTROPHIC DERMAL FIBROBLASTS AND TISSUES AFTER INTERFERON TREATMENT

BY

RIJIAN WANG ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement for the degree of **DOCTOR OF PHILOSOPHY**.

IN

EXPERIMENTAL SURGERY

DEPARTMENT OF SURGERY

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **"Interferons and Dermal Fibrotic Disorders: Nitric Oxide Production and Transforming Growth Factor-β1 Gene Expression by Normal and Hypertrophic Dermal Fibroblasts and Tissues after Interferon Treatment"** submitted by **Rijian Wang** in partial fulfillment for the degree of **DOCTOR OF PHILOSOPHY IN EXPERIMENTAL SURGERY.**

Dr. Edward E. Tredget

Dr. Aziz Ghahary

Paul Scott
Dr. Paul G. Scott

Kenneth G. Roy
Dr. Kenneth Roy

Dr. Richard Schulz

Gregory S Schultz
Dr. Gregory E. Schultz

DATE: Nov. 25, 1997

ABSTRACT

Accumulating evidence indicates that cytokines and other bioactive molecules produced by leukocytes and fibroblasts play a critical role in regulation of wound healing and scar remodeling. Among the cytokines involved in wound healing and hypertrophic scar (HSc) formation, transforming growth factor-beta (TGF-β) and interferons are important in that they appear to act in opposition in regulating extracellular matrix metabolism. Among the other bioactive molecules produced by living cells, nitric oxide (NO) seems to be of special interest due to its functional activities in collagen synthesis and degradation. This study was designed to obtain more information about the involvement of these molecules in wound healing and HSc formation.

It was shown that human dermal fibroblasts express both endothelial and inducible NO synthases (NOS) and produce NO in vitro. These results indicate that fibroblasts, like platelets and leukocytes, are involved in the inflammatory response and that NO may play a role in the wound healing process. Furthermore, it was found that HSc fibroblasts produce less NO and express less endothelial NOS than normal cells, suggesting that NO may be involved in HSc formation. Interestingly, HSc tissues also possess less endothelial NOS protein than normal skin, indicating that NO may participate in regulation of HSc formation in vivo.

A new procedure was developed to construct gene-specific internal standards for quantitative reverse transcription-polymerase chain reaction (RT-PCR) to quantify TGF-β1 gene expression in small numbers of cells or small amounts of tissue. This method was validated by Northern analysis for TGF-β1 mRNA and supported by ELISA for TGF-β protein production by dermal

fibroblasts. The results showed that HSc fibroblasts and tissues express more TGF-β1 mRNA and protein than normal controls, suggesting that overexpression of TGF-β by fibroblasts in tissues may be responsible for HSc formation. Autoinduction of TGF-β may be one of the mechanisms for the over-expression of TGF-β in HSc cells and tissues. Treatment of fibroblasts with TGF-β stimulates their proliferation and collagen synthesis, while treatment of the cells with interferon-α2b (IFN-α2b) and -γ inhibits proliferation and collagen synthesis. It is of interest that IFN reduces TGF-β mRNA expression and protein synthesis. Treatment of HSc patients with IFN-α2b also reduces TGF-β mRNA in the tissues and protein in the serum. These observations indicate opposite actions for TGF-β and IFNs in the regulation of extracellular matrix metabolism.

In conclusion, human dermal fibroblasts synthesize NO and this is enhanced by IFN-γ and bacterial lipopolysaccharide. HSc fibroblasts and tissues express more TGF-β but less NO. TGF-β stimulates whereas IFNs inhibit fibroblast proliferation and collagen synthesis. The inhibitory action of IFNs on TGF-β expression and their antagonistic effects of IFNs on TGF-β-stimulated cell proliferation and collagen synthesis indicate a new mechanism for the anti-fibrogenic activities of IFNs, i. e., IFNs block the autocrine effects and autoinduction of TGF-β.

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LIST OF ABBREVIATIONS

bFGF	basic fibroblast growth factor
bp	base pair
C_{1q}	component 1q of complement
CaM	calmodulin
cdk	cyclin-dependent kinase
eNOS	endothelial nitric oxide synthase
DMEM	Dulbecco's modified Eagle medium
cDNA	complementary deoxyribonucleic acid
ECM	extracellular matrix
EGF	epidermal growth factor
EGTA	ethylene glycol-bis (beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescent activated cell sorting
FCS	fetal calf serum
GAG	glycosaminoglycan
GAF	gamma-interferon activated fragment
GAS	gamma-interferon activated sequences
GITC	guanidinium isothiocyanate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HSc	hypertrophic scar
IFN-γ	interferon-γ
IGF-1	insulin-like growth factor-1
IL-1	interleukin-1
iNOS	inducible nitric oxide synthase
ISGE	interferon-stimulated gene element
ISGF-3	IFN-α-stimulated gene factor 3 complex
JAK1	Janus kinase 1
LAP	latency associated peptide

LPS	lipopolysaccharide
mAb	monoclonal antibody
Mad	Mothers against dpp
MAPK	mitogen-activated protein kinase
mRNA	messenger ribonucleic acid
MW	molecular weight
NADPH	nicotinamide-adenine dinucleotide phosphate (reduced form)
NF-κB	nuclear factor-kappa B
NMMA	N^G-monomethyl-L-arginine
NO	nitric oxide
PDGF	platelet-derived growth factor
RT-PCR	reverse transcription-polymerase chain reaction
SEM	standard error of the mean
SH	Src homology
STAT	signal transducers and activators of transcription
TBSA	total body surface area
TGF-β	transforming growth factor-beta
TIMP	tissue inhibitor of metalloproteinases
TNF	tumor necrosis factor

CHAPTER ONE

GENERAL INTRODUCTION

1.1 INTRODUCTION

1.1.1 Wound Healing and Scar Formation

Wound healing is a very complex process including cell activation, migration, differentiation, proliferation, and production of a variety of proteins and other molecules besides the regular metabolic wastes (Bennett and Schultz, 1993; Levenson and Demetriou, 1992; Martin and Peacock, 1992). The major proteins and proteoglycans produced by cells in the healing wounds include thrombotic and thrombolytic factors, extracellular matrix proteins, enzymes, and cytokines (Wahl and Wahl, 1992). The other major molecules produced by cells in the wound include oxygen metabolites and free radicals such as superoxide anion, hydrogen peroxide, hydroxyl radical, and nitric oxide (NO), steroids such as glucocorticoids, and eicosanoids such as leukotrienes and prostaglandins (Robson and Heggers, 1992). These molecules, working in concert or in sequence, participate in and guide the wound healing process (Bennett and Schultz, 1993; Martin and Peacock, 1992). The wound healing process is finely regulated under normal conditions, in which cytokines and the other molecules play an essential role (Bennett and Schultz, 1993; Martin and Peacock, 1992). Proliferation and migration of cells, deposition of extracellular matrix proteins and formation of new blood vessels result in scar formation. The result of this type of condition is a normal time span for the healing process and a normal scar.

Under abnormal conditions such as severe thermal injury or trauma, bacterial infections, and wounds complicated by other diseases, the wound healing process changes since additional factors, such as depletion or over-production of cytokines and accumulation of toxic metabolites, are involved in the process (Hunt and Hussain, 1992). One of the common consequences is a delayed wound healing and non-healing wounds such as those found in diabetic or cancerous patients (Lawrence, 1992; Mast, 1992). On the other hand, some patients undergo an enhanced wound healing process, characterized by excessive neovascularization and granulation as well as excessive collagen synthesis and deposition by fibroblasts, resulting in the development of hypertrophic scar (HSc) or keloid under certain circumstances (Boykin and Molnar, 1992).

1.1.2 Clinical Problem and Hypertrophic Scar Development

Hypertrophic scarring, a fibroproliferative disorder of the dermis, frequently occurs after wounding, particularly after severe thermal injury of the skin (Murray and Pinnell, 1992; Scott et al, 1994; Tredget et al, 1997). Clinically, HSc is characterized by raised, erythematous, itchy, nodular and inelastic scars that undergo slow and incomplete regression in comparison to normal scars. It frequently causes severe cosmetic and functional impairment (Scott et al, 1994; Tredget et al, 1997). Unlike keloid, which more likely occurs within the black population and involves surrounding normal tissues, HSc is confined to the injured area (Murray and Pinnell, 1992). Pathologically, HSc is characterized by excess collagen synthesis and deposition by fibroblasts, over-proliferation of fibroblasts, excess vascularity and infiltration of immune cells such as lymphocytes, macrophages and mast cells (Murray and Pinnell, 1992; Scott et al, 1994; Tredget et al, 1997).

1.1.3 Factors Responsible for and the Mechanisms of HSc Formation

Many factors are implicated in HSc formation. These include age, gender, race or genetic factors, healing conditions and location of the wounds (Scott et al, 1994). Delayed epithelization of the wound due to lack of sufficient skin grafts for wound coverage may also be responsible for HSc formation. Prolonged exposure of subcutaneous tissue may trigger over-production of fibrogenic and angiogenic cytokines by the cells in the tissue (Boykin and Molnar, 1992).

It is possible that multiple factors work in concert to promote HSc. The synergistic effects of cytokines *in vitro* support this assumption (Bennett and Schultz, 1993). For example, transforming growth factor-beta (TGF-β) is more fibrogenic and less mitogenic for fibroblasts (Roberts et al, 1986), whereas for insulin-like growth factor-1 (IGF-1) the reverse is true *in vitro* (Bennett and Schultz, 1993; Bird and Tyler, 1994; Davidson et al, 1993). If both cytokines are given to cultured fibroblasts, both enhanced fibrogenic and mitogenic effects have been observed indicating a synergistic action of these cytokines (Bennett and Schultz, 1993). Another example are the synergistic effects observed with basic fibroblast growth factor (bFGF) and TGF-β (Davidson et al, 1993). More than two cytokines in cultures produce even more complex effects, which are difficult to interpret at present (Bennett and Schultz, 1993).

Another possible mechanism for HSc formation may be that the negative regulators of fibroblast proliferation and activation are down-regulated or missing. It has been shown that type I interferons (IFN-α, -β; Tredget et al, 1993) and type II IFN (IFN-γ; Harrop et al, 1995) down-regulate collagen synthesis and stimulate collagenase production while increased collagen production and decreased collagenase expression is observed in HSc. These findings suggest that IFNs may be down-regulated in HSc. The anti-fibrogenic effects of IFNs have been well-characterized and clinical application of IFNs for treatment of fibroproliferative disorders such as hepatic cirrhosis has been reported (Brenner and Alcorn, 1990; Castilla et al, 1991). With regard to the effects of NO on HSc formation, the findings that HSc fibroblasts and tissues produce less NO and express less NO synthase (NOS) suggest an important role of NO in the formation of HSc (Wang et al, 1997).

Hypertrophic scarring is a serious problem for reconstructive surgery and rehabilitative medicine. There is no effective treatment for HSc and keloid although several procedures including surgical and non-surgical methods have been used (Murray and Pinnell, 1992). Surgical operation is often necessary to relieve serious functional impairment and often includes full or partial-thickness skin-grafting. There are many limitations for surgical operations (Murray and Pinnell, 1992), of which lack of skin graft in the case of large scars is the most important limitation. Multiple operations are required for large scars in certain location (Murray and Pinnell, 1992). Recurrence of HSc after operation in some patients is another limitation (Murray and Pinnell, 1992). The non-surgical procedures, including pressure-garment therapy, silicone gel sheets, topical application and intralesional injection of glucocorticoids, are not very effective or are often limited by their side-effects. Since there is no very effective treatment for HSc, it is very important to understand the etiology and mechanisms of HSc formation so as to prevent it instead of treating it. The purpose of this thesis is to gain more information about the etiology and pathophysiology of HSc in order to develop more efficient procedures to prevent the formation of this dermal fibroproliferative disorder.

1.2 THESIS OUTLINE

The research described in this thesis was designed and conducted to examine two separate but closely related aspects of the physiology and gene expression of dermal fibroblasts, which are major players in HSc formation. The first aspect of the research was to examine NO production and NOS expression by dermal fibroblasts and to compare this in fibroblasts derived from HSc tissue and normal skin. The second aspect of the research was to examine TGF-β1 mRNA synthesis and protein secretion by normal and HSc dermal fibroblasts and to examine TGF-β1 gene expression after IFN treatment.

Chapter Two is a review of literature relevant to the research project. Chapter Three describes the findings that human dermal fibroblasts produce NO and express both constitutive and inducible NOS isoforms. In Chapter Four I compare NO production and NOS expression between dermal fibroblasts derived from HSc tissue and from normal skin. Chapter Five describes a new procedure for constructing gene-specific internal standards for reverse transcription-polymerase chain reaction (RT-PCR) to quantify TGF-β1 mRNA expression by HSc and normal fibroblasts and tissues. Chapter Six describes the results for cell proliferation, collagen synthesis, TGF-β1 mRNA and protein production by HSc and normal fibroblasts and tissue after IFN treatment. The final chapter is a synopsis of the thesis and general discussion of the findings of the research project.

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CHAPTER TWO

LITERATURE REVIEW

2.1 WOUND HEALING: AN ESSENTIAL PROCESS FOR SURVIVAL

2.1.1 The Function and Structure of Skin

The integrity of the skin is essential for survival of all animals including human beings living in a hostile and unpredictable environment. The skin has a surface area of 1.5 to 2 m^2 for an average adult, making it the largest organ (Mast, 1992). The principal functions for the skin are protection and thermal regulation. It prevents dehydration through evaporative water loss and acts as a mechanical barrier against a variety of insults such as chemicals and infectious agents (Mast, 1992). The thermal regulatory function of the skin operates through the control of blood flow in the dermis and through sweating in human beings and a few other mammals. Anatomically, the skin is divided into two layers: the epidermis and the dermis. The epidermis is the superficial and protective layer made up of a keratinized, stratified, squamous epithelium. The dermis is subdivided into two layers: the papillary dermis and the reticular dermis. The more superficial papillary dermis contains a rich supply of blood and lymph vessels and numerous nerve endings. The deeper reticular dermis is mainly composed of a layer of connective tissue providing the structural support for the skin. Fibroblasts and the extracellular matrix (ECM) proteins they synthesized are the major components of this layer in which the dermal appendages such as hair follicles and sweat glands are located (Mast, 1992). The subcutaneous tissue, deeper to the dermis, contains a variety of cells including fibroblasts, histiocytes, adipocytes, and immune cells such as lymphocytes, monocytes, dendritic cells and mast cells. These cells are responsible for structural integrity and metabolism of the dermis, storage of energy, foreign antigen processing and cytokine production after wounding (Mast, 1992).

2.1.2 The Normal Healing Response in Dermal Wounds

The wound healing process is a very complex series of events that involves both cellular and ECM components. It is initiated at the precise moment when tissue injury occurs (Mast, 1992). The duration of the wound healing process varies depending on severity, site and condition of the wounds. For descriptive purposes, this process can be simplified to four sequential but overlapping stages: hemostasis, inflammation, proliferation, and remodeling (Schilling, 1976).

Hemostasis: Upon disruption of endothelial integrity of the blood vessels during tissue injury, peripheral blood cells spill into the wound site, where the platelets come into contact with damaged collagen and other tissue debris, triggering the aggregation and degranulation process. The released alpha and dense granules contain peptide growth factors and thrombotic factors, which cause fibrin deposition and polymerization and continued aggregation of platelets, resulting in the formation of a thrombus. Hemostasis is achieved by the formation of a thrombus within the wound in conjunction with vasoconstriction triggered by trauma. The factors released by activated platelets responsible for inflammation include fibronectin, serotonin, adenosine diphosphate, platelet-activating factor, thromboxane A_2 and gelatinase (Weksler, 1988; Sawicki et al, 1997). The peptide growth factors released by platelets include epidermal growth factor (EGF; Oka and Orth, 1983) platelet-derived growth factor (PDGF; Ross et al, 1974), IGF-1 (Karey and Sirbasku, 1989), and TGF-β (Assoian et al, 1983). PDGF and TGF-β are chemoattractants for fibroblasts, smooth muscle cells and inflammatory cells, leading to extravasation of these cells (Mast, 1992).

Inflammation Phase: The initiation of acute inflammation occurs immediately after thrombus formation and secondary vasodilation. Neutrophils comprise the major cell population during the early stage of acute inflammation (Mast, 1992), followed by monocyte infiltration. Neutrophils actively participate in the inflammatory response and protect the wound against bacterial infection by release of degrading enzymes such as elastase, collagenase and other hydrolytic enzymes and bactericidal molecules such as defensins (acid-extractable, arginine-and cystine-rich peptides with antimicrobial properties; Ganz et al, 1985; Selsted et al, 1985), superoxyde anion, H_2O_2, hydroxyl radical, and NO (Wahl and Wahl, 1992; Yui et al, 1991). These degradative enzymes and oxygen metabolites are also responsible for neutrophil-induced tissue damage during inflammation (Wahl and Wahl, 1992). Neutrophils also protect the wound against bacterial infection by phagocytosis of bacterial and tissue debris (Wahl and Wahl, 1992).

The circulating monocytes are converted into macrophages as they enter the wound, where they destroy bacteria and debride the wound by phagocytosis. Besides their phagocytic function, the wound macrophages are major sources of cytokines and growth factors, such as TGF-β, IGF-1, interleukin-1 (IL-1), IL-6, and tumor necrosis factor (TNF), which are important in wound healing (Diegelmann et al, 1981; Roberts et al, 1986). Another important cell population in the healing wound are lymphocytes whose role is not fully understood at present (Mast, 1992). However, lymphokines such as IL-1, TGF-β, TNF-α and IFN-γ, produced by activated lymphocytes, are important in wound healing (Barbul, 1992).

Proliferation Phase: This phase is also known as the phase of fibroplasia or granulation tissue formation. Fibroblasts play a critical role in this phase although their activity is initiated and regulated by cytokines and growth factors produced by activated platelets and infiltrated macrophages and lymphocytes (Mast, 1992). Activated fibroblasts migrate to the wound site, proliferate and produce collagens and other ECM proteins as well as cytokines and growth factors. Collagen synthesis and deposition by fibroblasts are prominent at this phase. This is coincident with the degradation of proteoglycans produced in the hemostatic stage of wound healing. Proliferation and migration of endothelial and epithelial cells also occur at this stage as neovascularization and epithelization proceed (Mast, 1992). These processes lead to wound closure and scar formation.

Remodeling Phase: The remodeling process starts late during the proliferation phase and persists for months to years. During the early part of this phase, the abundant neovascularization of early granulation tissue recedes followed by the principal processes, the synthesis and degradation of collagen and the formation of mature scar (Mast, 1992). During this phase, the tensile strength of the wound continues to increase despite a reduction in collagen synthesis. The structural modification of the newly synthesized collagen leads to the gain in strength of the wound (Miller and Gay, 1992). Crosslinking of collagen fibrils is mainly responsible for these modifications. However, this process is not perfect since the remodeled collagen never achieves the normal structure of the dermal collagen (Mast, 1992).

2.1.3 Factors That Influence Wound Healing

A variety of conditions affect the wound healing process as a consequence of the complexity and multiple participants in wound repair. Bacterial infection retards or even reverses the healing process. The delayed wound healing due to bacterial infection is caused by local destruction of tissue by bacterial growth and enzymatic action and prolongation of the inflammatory phase of healing (Mast, 1992). Bacterial infection also causes massive infiltration and activation of lymphocytes and macrophages, leading to over-production of cytokines, especially fibrogenic cytokines such as TGF-β1 and IL-1 responsible for later HSc formation.

Nutrition is very important for wound healing due to the higher demand for protein synthesis to rebuild the damaged tissue during wound repair. Essential amino acids are the most important nutrients due to the higher rate of protein synthesis during wound healing. Other nutrients such as vitamins, fatty acids, carbohydrates, trace elements, and hormones are also important (Levenson and Demetriou, 1992). Deficiency of these nutrients causes delayed wound healing. Some diseases, such as diabetes and cancer, and medications, such as glucocorticoids and immunosuppressive drugs, also retard wound healing (Levenson and Demetriou, 1992). On the other hand, some factors enhance the wound healing process by directly or indirectly stimulating epithelial cells, endothelial cells and fibroblasts to proliferate and to synthesize proteins. These factors include bacterial products such as a peptidoglycan derived from Staphylococcus aureus (Castaldi et al, 1996; Kilcullen et al, 1996) and some fibrogenic cytokines such as TGF-β, PDGF (Roberts et al, 1986), FGFs (Bennett and Schultz, 1993), and fibroblast activating factor (Bennett and Schultz, 1993). Over production of these growth factors could be a consequence of other conditions such as bacterial infection of the wound (Robson and Heggers, 1992) or hypoxia (Falanga et al, 1991). The combination of these factors may be responsible for HSc formation.

2.2 HYPERTROPHIC SCARRING: PATHOLOGICAL WOUND HEALING

2.2.1 Pathology and Clinical Aspects

Hypertrophic scarring is commonly seen after severe thermal injury of the skin although it can happen after any form of traumatic injury that penetrates the dermis (Scott et al, 1994; Tredget et al, 1997). HSc can be classified as a fibroproliferative disorder. Other examples include keloid, hepatic cirrhosis, pulmonary fibrosis, atherosclerosis, scleroderma, and rheumatoid arthritis (Bitterman and Henke, 1991; Rockwell et al, 1989). These fibroproliferative conditions have common or similar characteristics and pathology although each has its own specific clinical manifestations and symptoms. Some of them such as hepatic cirrhosis, pulmonary fibrosis and atherosclerosis often result in death of the victim (Bitterman and Henke, 1991; Rockwell et al, 1989) while others such as HSc, keloid and rheumatoid arthritis often cause other serious clinical problems (Scott et al, 1994; Tredget et al, 1997).

From a histopathological point of view, HSc is characterized by an increase in thickness of the epidermis due to over-proliferation of keratinocytes and dermis due to excessive collagen deposition by fibroblasts (Tredget et al, 1997). The epithelial ridges are missing due to an increase in volume of the dermis and flattening of the epidermis (Murray and Pinnell, 1992; Tredget et al, 1997).

Collagen nodules are apparent in HSc but not in normal wound healing. These nodules contain numerous fibroblasts and collagen fibrils, arranged in swirl-like clusters (Linares et al, 1972). The HSc lesions have increased vascularity and a moderate perivascular leukocyte infiltration, which contains a moderate numbers of mast cells, plasma cells and lymphocytes (Kischer and Bailey, 1972).

From the clinical point of view, HSc is characterized by raised, erythematous, itchy, fibrous lesions that undergo incomplete regression over a wide range of times (Tredget et al, 1997). Dependent on the severity and location of the scars, the clinical manifestations are highly variable. Cosmetic and functional impairment is the most common complaint of HSc patients, along with intense itchiness. Cosmetic disfigurement often causes psychological problems and affects the social activity of the patients. This may lead to the development of stress-related disorders such as sensitivity to infection, digestive system disorders, etc. Functional impairment is very common in patients with severe HSc and is fundamentally dependent on the location and the severity of the scar. The most important functional impairment is the restriction of joint movement, especially at the locations that require free and precise movement, such as hands, neck, arms, and legs.

Although many approaches have been proposed, treatment of HSc remains a challenge for plastic surgeons since it is time-consuming, expensive, and there are few consistently successful approaches (Tredget et al, 1997). A high rate of recurrence has been observed in surgical excision of HSc without adjuvant therapy (Tredget et al, 1997). This, along with frequent requirement for full-thickness skin graft or flaps in large scars potentiates the difficulty of using surgical therapy for HSc. Conservative therapies, which include the use of pressure garments, silicone gel sheets, conformers, serial casting and splints, have limited success, and often require prolonged, continuous application of the devices (Tredget et al, 1997). Intralesional injections of corticosteroids are limited by their marginal therapeutic effects and marked systemic side-effects. Therapeutic approaches using modern technology such as laser therapy, intralesional injection of calcium channel blockers (verapamil) or calmodulin inhibitor (trifluoperazine) have been used in clinics to treat HSc (Tredget et al, 1997). However, the small number of cases reported for these newly developed approaches are the major drawbacks because it is hard to evaluate the benefits and side-effects (Tredget et al, 1997). Collectively, none of the therapeutic approaches is entirely satisfactory. Better understanding of the etiology, pathogenesis, and molecular mechanisms of HSc formation would be helpful to develop new preventive and therapeutic approaches for this fibroproliferative disorder.

2.2.2 Etiology of Hypertrophic Scarring

Although many risk factors such as age, gender, genetic factors, location of the wound, and prolonged inflammation phase in burn wounds are recognized, the etiology of HSc formation is not clear at present (Scott et al, 1994; Tredget et al, 1997). It has been observed that young children more often develop severe HSc in comparison to adults after thermal injury of the skin (Boykin and Molnar, 1992; Mast, 1992). This may be the result of higher levels of growth related hormones such as growth hormone and IGF-1 and lower levels of sex steroids in young children. Growth hormone has been shown to accelerate wound healing by exerting its anabolic effects on a variety of cells and by its mitogenic and stimulatory effects on leukocytes such as neutrophils, macrophages and lymphocytes (Fornari et al, 1994). IGF-1 has been shown to be mitogenic for and to have fibrogenic effects on fibroblasts (Bird and Tyler, 1994). Sex hormones, especially female sex steroids, are inhibitory for leukocytes (Feinberg et al, 1992; Wang and Belosevic, 1995a; 1995b). They directly inhibit macrophage activation and lymphocyte proliferation, resulting in a reduced inflammatory response and lower cytokine production by the cells (Polan et al, 1989; Wang and Belosevic, 1995a; 1995b). The lower levels of sex steroids in young children may lead to higher levels of fibrogenic cytokines resulting in HSc formation. This is further demonstrated in gender differences in HSc formation. Male burn victims are prone to develop more severe HSc than females as seen in the burn clinics. Although there is no report regarding the effect of sex steroids on fibroblast function, the effects of sex steroids on the functional activity of leukocytes have been observed by many investigators (Benten et al, 1992; Feiberg et al, 1992; Magnusson and Fossum, 1992; Wang and Belosevic, 1995a; 1995b). Furthermore, leukocytes such as lymphocytes and macrophages also possess receptors for sex steroids, suggesting that these cells respond to sex steroids through the classic pathway of steroids (Cohen et al, 1983; Jakob et al, 1992). It is likely that sex steroids affect wound healing and HSc formation through their regulatory effects on fibrogenic cytokines produced by leukocytes.

The delayed epithelization of the wound due to lack of sufficient skin grafts for wound coverage is also responsible for HSc formation. Prolonged exposure of subcutaneous tissue may trigger over-production of fibrogenic and angiogenic cytokines by the cells in the tissue (Boykin and Molnar, 1992). Bacterial infection of the wound is another factor responsible for HSc formation.

Bacterial products such as lipopolysaccharide (LPS) are directly stimulatory and chemotactic to leukocytes, which are major sources of fibrogenic and angiogenic cytokines (Mast, 1992). Immune responses to the infection enhance the cell infiltration and cytokine production. This is beneficial for the host to eliminate bacterial infection but may result in over-production of fibrogenic cytokines and consequently, HSc formation (Mast, 1992). It has been demonstrated that the exaggerated inflammation in delayed healing wounds leads to a high rate of HSc formation independent of age, racial background and the site of wound (Deitch et al, 1983). Genetic factors are also important for HSc formation. This is indicated by the more common and severe HSc and keloid development in black people. However, the genes responsible for HSc formation are not identified yet (Murray and Pinnell, 1992).

2.2.3 Heterogeneity of Fibroblasts in Hypertrophic Scarring

Heterogeneity has been observed in mouse and rat fibroblasts (Fries et al, 1994), in human gingival fibroblasts (Hou and Yaeger , 1993; Phipps et al, 1997), in myofibroblasts (Gabbiani, 1994; Schmitt-Graff et al, 1994), and in fibroblasts derived from scleroderma patients (Jelaska et al, 1996; Torry et al, 1994). The subpopulations of fibroblasts have different characteristics in proliferation, cell surface marker (e. g. Thy-1) expression, collagen synthesis, collagenase production (Fries et al, 1994), glycosaminoglycan (GAG) synthesis (Pagliarini et al, 1995), and sensitivity to radiation (Dileto and Travis, 1996; Ma et al, 1996). The heterogeneity of fibroblasts may be essential for the plentiful functions of fibroblasts distributed throughout different organ systems of the body.

Heterogeneity has also been observed in human dermal fibroblasts. It has been reported that fibroblasts derived from HSc express more mRNA for type I and type III procollagens and synthesize more collagen than those derived from normal skin (Ghahary et al, 1993). Collagenase production and mRNA expression is reduced in HSc fibroblasts (Ghahary et al, 1996). The increased collagen synthesis and decreased collagenase production plus enhanced IGF-1 production by HSc fibroblasts (Ghahary et al, 1995) may lead to over-deposition of collagen in the scar tissue, resulting in HSc formation (Ghahary et al, 1993). Recently, it has been demonstrated that HSc fibroblasts express less mRNA for endothelial NOS and produce less NO in comparison to normal fibroblasts, providing another piece of evidence of heterogeneity for human dermal fibroblasts (Wang et al, 1997).

2.3 EXTRACELLULAR MATRIX: FUNDAMENTAL ARCHITECTURE FOR MORPHOLOGY AND METABOLISM

2.3.1 Fibroblast Activation and Collagen Biosynthesis

The fibroblast is an important cell in most animal tissues. Under normal conditions, fibroblasts are sparsely distributed and usually quiescent throughout the connective tissue matrix (Morgan and Pledger, 1992). They are responsible for tissue integrity and ECM metabolism by producing structural (e. g. collagens) and functional (e. g. collagenase) proteins of ECM. The production and degradation of ECM are maintained in balance (Jeffrey, 1992). In damaged tissue, fibroblasts actively migrate towards the wound sites, proliferate, and produce more ECM proteins to facilitate wound healing. Activated fibroblasts in injured tissues also produce cytokines such as IL-1 (di Giovine and Duff, 1990; Oppenheim, 1986) and IL-6 (Willis et al, 1994), which further augment the inflammatory response of the tissue. On the other hand, activated fibroblasts produce IFN-β and -α (Morgan and Pledger, 1992; Oppenheim, 1986), which act as autocrine growth inhibitors to regulate the proliferation of fibroblasts (Morgan and Pledger, 1992).

Collagen is the major product of fibroblasts and its biosynthesis is regulated by a variety of molecules including cytokines (Morgan and Pledger, 1992). Collagen is an obligatory component of ECM and connective tissue for all multicellular organisms, in which the aggregates of collagen molecules throughout the tissue are responsible for establishing and maintaining the physical integrity of diverse extracellular structures and contributing to the functional capabilities of the animal as a whole (Miller and Gay, 1992).

The molecular structure and folding of collagen is unique. It contains lengthy domains of triple-helical conformation that is only shared by a few other molecules such as C1q (Kilcherr et al, 1985), the asymmetric form of acetylcholinesterase (Mays and Rosenberry, 1981), the apoprotein of pulmonary surfactant (Benson et al, 1985; Floros et al, 1986), and the mannose-binding proteins in serum and liver (Drickamer et al, 1986). The primary structure of collagen polypeptide chains contains the repeated Gly-X-Y sequence, in which glycine occurs in every third position along the chain. This is absolutely required for the triple helix since there is no space within the structure for side chains other than glycine at every third residue (Miller and Gay, 1992). In vertebrates, a total of at least 21 types of collagen has been recognized on the bases of their structures and distribution (Morgan and Pledger, 1992). They are collectively composed of as many as 25 unique polypeptide chains, which are divided into 5 groups, designated as α1 to α5 (Miller and Gay, 1992). Type I collagen, which is composed of two α1 chains and a single α2 chain, is the most abundant triple helix protein in human dermis (Miller and Gay, 1992).

The dynamic synthesis and degradation of interstitial and basement membrane collagens are fundamental processes involved in growth, development, morphogenesis, repair, and remodeling under physiological and pathological conditions (Phillips and Wenstrup, 1992). The balance of collagen synthesis and degradation is maintained by many factors including peptide cytokines (Bennett and Schultz, 1993; Robson and Heggers, 1992) and other non-peptide molecules such as steroids, eicosanoids, and NO (Murrell et al, 1995; Robson and Heggers, 1992). Following injury, the rate of collagen turnover is increased due to synthesis and activation of collagenases. This increased collagen turnover facilitates wound repair and remodeling. Under certain conditions, the balance of collagen synthesis and degradation may be disturbed due to overproduction or depletion of certain cytokines or other regulatory molecules resulting in delayed wound healing or HSc formation.

2.3.2 Collagenases and Collagen Degradation

Degradation of native fibrillar collagen requires a class of specific proteinases collectively called collagenases, which belong to the family of extracellular metalloproteinases that include collagenase, gelatinase, and stromelysin (Jeffrey, 1992). These enzymes are capable of degrading connective tissue components and require zinc as a cofactor, which is tightly bound to the protein at the active site (Jeffrey, 1992). Collagenase cleaves the native collagen molecule at one locus, resulting in two triple helical fragments termed TC^A and TC^B, which are unstable at 37°C and therefore denatured. The complete degradation of collagen depends on gelatinase and other proteinases. Gelatinase degrades denatured collagens and native type IV as well as native type V collagen. Stromelysin has a wide specificity for connective tissue proteins that includes fibronectin, laminin, native type IV collagen and proteoglycan from cartilage (Quinones et al, 1990).

As a class, all the collagenases have several common properties. Besides the use of zinc as a co-factor shared by other metalloproteases, all collagenases require calcium for activity (Seltzer et al, 1977). Although the exact role of calcium is not known, preliminary evidence suggests that it is required for conformational stability of these enzymes since in the absence of these cations, collagenases have less thermal stability and are more sensitive to proteinase digestion (Seltzer et al, 1977). Another property for collagenases is that they all catalyze the identical cleavage in the type I collagen molecule. The site is at the peptide bond joining residues 775- and 776- in the three chains composing the triple helices (Miller et al, 1976). The amino acids at this site are glycyl-isoleucine ($\alpha 1$ chains) and glycyl-leucine ($\alpha 2$ chain), which have been completely conserved throughout evolution, from tadpole tail type I collagen to human skin type I collagen (Jeffrey, 1992). This highly conserved collagen degradation mechanism argues for the importance of precise degradation of the most common and the toughest structural protein, collagen (Jeffrey, 1992). However, collagenases derived from different cell types within a single species are quite different in their molecular properties (Hasty et al, 1984). For example, collagenase from human fibroblasts is different from that derived from neutrophils in terms of molecular structure and antigenicity. Human fibroblasts seem to synthesize their collagenase upon biological demand while neutrophils simply store the synthesized collagenase in their neutral granules. Nevertheless, these two collagenases do cleave the same peptide bond (Hasty et al, 1984; 1986). This difference in structural property and synthesis profile but equivalence in enzymatic properties may ensure the physiological function of the cells without affecting the requirement for precise collagen degradation. The physiological significance of this difference between fibroblast collagenase and neutrophil collagenase is not fully understood. This difference might be of great significance in wound healing.

Human fibroblast collagenase is synthesized and secreted as an inactive proenzyme procollagenase with a molecular mass of approximately 52 kDa (Jeffrey, 1992; Stricklin et al, 1977; 1978). The zymogen can be activated *in vitro* by a variety of reagents which remove a "pro-" piece of peptide about 10 kDa in the procollagenase molecules. The most commonly used procedure to activate procollagenases *in vitro* is to treat the proenzyme with trypsin for a "limited" cleavage. It is assumed that removal of this "pro-" piece from procollagenase molecules causes a conformational modification that results in exposure of the active site or proteolytic domain (Birkedal-Hansen et al, 1976; Jeffrey, 1992). This *in vitro* model of procollagenase activation provides a temporarily satisfying mechanism for the *in vivo* procollagenase activation, although the active form of collagenase has never been firmly established in any tissue. This is due to the spontaneous degradation of active form of collagenase and the difficulty to remove and purify the small amount of enzyme from tissue collagen fibrils (Jeffrey, 1992).

Collagenase has an unusual characteristic with important physiological significance: once bound to the molecules within a collagen fibril, the enzyme seems to move from the cleaved molecule to an intact molecule within that fibril without an intervening dissociation step. It will continue to cleave the molecules until the substrate has been completely degraded (Welgus et al, 1980). This situation is unacceptable for the precise degradation mechanism in collagen turnover, raising the possibility that other mechanism(s) must operate to ensure the properly maintained architecture of the ECM. This leads to the discovery of tissue inhibitor of metalloproteinases (TIMP), a class of glycoproteins with molecular mass of approximately 30 kDa (Jeffrey, 1992; Stricklin and Welgus, 1983). The inhibitor is capable of forming extremely tight complexes with collagenases and inhibits their enzymatic activity, resulting in the formation of sharp geographical boundaries of collagenolytic activity and the protection of connective tissue from the activity of the enzyme (Jeffrey, 1992; Welgus and Stricklin, 1983). In this way, the cells regulate collagenolysis in the tissue to preserve the normal collagen matrix surrounding the wound sites, where the collagenases are usually synthesized and activated.

2.3.3 Other ECM Structural Molecules Involved in Wound Healing

Besides collagens, ECM contains a variety of structural molecules that serve as "ground substance" for ECM, as accessory molecules for collagen fibrils, as molecular links for signal transduction, and as adhesion ligands or receptors for cells to attach to substratum (Grinnell, 1992). These molecules include proteoglycans, hyaluronic acid, elastin, and other ECM glycoproteins.

Proteoglycans: A proteoglycan is a protein-polysaccharide complex that contains a protein core and one or more covalently bound polysaccharides known as glycosaminoglycans (GAGs). Hyaluronic acid, also known as hyaluronan, is a GAG that consists of a large linear polysaccharide without sulfate groups which is not covalently bound to proteins to form proteoglycans (Weitzhandler and Bernfield, 1992). The GAG chains of proteoglycans consist of 4 types of sulfated polysaccharides, chondroitin sulfate, heparan sulfate, dermatan sulfate, and keratan sulfate. These GAGs differ in the configuration of their carboxylate groups, the number and positions of sulfate groups, and the connection between the two sugar molecules that make one disaccharide unit. The functional roles of proteoglycans are largely dependent on the molecular configuration such as the size and structure of the core proteins and the extent of glycosylation as well as the types of the GAG chains and the locations of the molecules, whether they are within intracellular granules, at the cell surface, or within the ECM (Weitzhandler and Bernfield, 1992). Within the intracellular granules, such as in mast cell secretory granules where the serine proteases and vasoactive amines are positively charged at physiological pH, the proteoglycans may act like an ion-exchange resin that binds and concentrates those positively charged molecules. Upon binding the proteoglycan-protease or proteoglycan-amine complexes may reversibly inactivate the intra-granular proteases to prevent nonspecific degradation of these useful molecules before secretion (Stevens et al, 1985). In the mammalian nervous system, heparan sulfate proteoglycans are involved in neuronal connections (Weitzhandler and Bernfield, 1992). At the cell surface, proteoglycans are involved in cell-matrix interaction and antigen presentation (Weitzhandler and Bernfield, 1992). Within the ECM, proteoglycans are responsible for the basement membrane permeability and to maintain the structures that are resistant to compression such as cartilage (Weitzhandler and Bernfield, 1992).

Depending on the molecular size of their core proteins, proteoglycans are roughly classified into two groups: large and small. The core proteins of large proteoglycans are usually 200 kDa or more. They are important for the structural integrity of the tissue where they are predominant. For example, aggrecan is a chondroitin sulfate proteoglycan predominantly found in cartilage. It has a core protein of about 200 kDa with attached GAG chains of chondroitin sulfate and keratan sulfate making its total molecular mass about ten times its core protein mass (Stevens et al, 1984). It usually forms large molecular aggregates noncovalently bound to hyaluronan. Due to its high density of GAG chains, aggrecan attracts large amounts of water, allowing it to increase the resiliency of cartilage and accounting for the main physical property of this proteoglycan (Hardingham and Fosang, 1992).

Perlecan, a heparan sulfate proteoglycan, is another member in this group distributed primarily in the basement membrane. Its core protein is about 470 kDa with 5 domains which are shaped into specific protein motifs having similarities to regions of known polypeptides, which are used in binding and delivering nutrients and lipids, cellular growth, and in intercellular signaling pathways (Iozzo et al, 1994; Iozzo and Murdoch, 1996). These 5 domains of the core protein have been assumed to have different functions, suggesting that this proteoglycan may be multi-functional.

Another large proteoglycan is versican, a chondroitin sulfate proteoglycan with a multi-domain core protein of 265 to 370 kDa. This molecule is highly glycosylated at the central region of the core protein leaving bare polypeptide domains at both ends. It has proposed that this configuration would keep versican molecules "stretched" to facilitate its functional activities (LeBaron et al, 1992). Versican has been considered to have several functions including cell adhesion, proliferation, differentiation and signal transduction, although its functional role is not well-defined. These functions are implied from the primary structure of the core protein (Zimmermann and Ruoslahti, 1989).

The core protein for small proteoglycans is around 40 kDa, but it can be as small as 10 kDa with a few polysaccharide chains attached. They can be grossly grouped into two classes: the serine-glycine repeat-containing molecules and the leucine-rich proteoglycans of the ECM (Iozzo and Murdoch, 1996; Kresse et al, 1994). Serglycin is a member of the serine-glycine repeat-containing proteoglycan whose core protein contains serine-glycine repeats, to which many chondroitin sulfate GAGs are attached. Serglycin is basically an intracellular granule proteoglycan that is implicated in functions such as enzyme inactivation, maintenance of electrical neutrality and proper osmotic pressure in the secretory granule of mast cells. It prevents natural killer cells from autolysis through its interaction with perforins in the cells (Matsumoto et al, 1995). All members of another family of small proteoglycans have a leucine-rich core protein and one or a few GAG chains. This family of proteoglycans include biglycan, decorin, fibromodulin, and lumican. Biglycan and decorin possess globular core proteins to which attached two and one chondroitin/dermatan sulfate GAG chain(s), respectively (Jarvelainen et al, 1991). The functional roles for biglycan and decorin are to bind to collagen fibrils to inhibit the lateral polymerization of collagen, modifying collagen fibril structure (Fleischmajer et al, 1991). Decorin has been observed to bind to TGF-β and to negatively regulate its activity (Yamaguchi et al, 1990). Fibromodulin, a keratan sulfate proteoglycan, has been reported to have inhibitory effect on collagen fibril formation (Plaas et al, 1990).

Lumican, a corneal keratan sulfate proteoglycan, was presumed to be responsible for corneal transparency (Blochberger et al, 1992; Cornuet et al, 1994).

Elastin: Elastin is the most insoluble and very elastic protein with distinct features which include the presence of desmosine and isodesmosine heterocyclic cross-links (Davidson et al, 1992) and the rigid α-helical regions along with the flexible hydrophobic repeats (Gray et al, 1973). The colorless elastin, electron-lucent component of ECM, along with the elastic fiber microfibrils, form the elastic fibers that can be visualized by electron microscopy (Davidson et al, 1992). The fundamental role of elastin in tissue is to act as a rubber-like material in an aqueous environment to provide the elastic recoil, which is critical for some tissues such as blood vessels, skin, the fascia of skeletal muscles, and the interstitium of the lung (Davidson et al, 1992). Elastin is synthesized and secreted primarily by mesenchymal cells such as vascular smooth muscle cells (Naranayan et al, 1976) and nuchal ligament fibroblasts (Mecham et al, 1981). Some types of epithelial (Rennard et al, 1984) and endothelial (Mecham et al, 1983) cells also have the capability of producing elastin after injury or *in vitro*, indicating their role in tissue repair. Elastin gene expression is regulated by a variety of factors including mechanical forces, presence of ECM, corticosteroids (Russell et al, 1995), and growth factors such as IGF-1, TGF-β, and basic FGF. The elastogenic properties of IGF-1 have been observed in arterial smooth muscle cells (Foster et al, 1987). It is of interest that TGF-β also enhances elastin synthesis by smooth muscle cells (Liu and Davidson, 1988) and fibroblasts (Sephel et al, 1987) and this elastogenic effect is antagonized by basic FGF and TGF-α (Davidson et al, 1993). These observations suggest that it is the balance between these hormonal growth factors that determine the elastin synthesis and degradation during tissue injury and repair (Davidson et al, 1995).

Extracellular Matrix Glycoproteins: The glycoproteins of ECM comprise a family of structural and adhesion molecules that are involved in wound healing. These include fibrin (fibrinogen), fibronectin, laminin, thrombospondin, and vitronectin. These molecules participate in the thrombotic and thrombolytic as well as the inflammatory responses by their chemoattractant properties (Grinnel, 1992). They serve as scaffolds for cell migration and also function as signal transduction and cell adhesion molecules. Among these ECM glycoproteins, fibronectin has been studied most thoroughly due to its biological significance in cell adhesion which is critical for the functional activity of cells during wound healing (Grinnell, 1992). The fibronectin molecule is composed of two identical polypeptides that are made up of three types of homologous repeating units. The molecule is the product of a single gene, but several different forms exist due to alternative splicing. The different forms of fibronectin may be responsible for its biological functions (Grinnell, 1992).

2.3.4 Cell Surface Glycoproteins Involved in Wound Healing

The cell surface glycoproteins, including the integrin family, selectin family, cadherin family, CD44, and some members of the immunoglobulin superfamily, are also important in wound healing. These molecules serve as adhesion receptors mediating cell-cell or cell-matrix adhesion by functioning as molecule anchors, and facilitate cell migration by functioning as dynamic cellular "feet" (Sonnenberg, 1993). They function as surface receptors for cells to collect information from ECM and mediate bidirectional transmembrane signal transduction (Dedhar and Hannigan, 1996). Of these cell surface glycoprotein receptors, integrins represent the largest known family of cell-adhesion molecules with complex functional activities in cell adhesion, migration, differentiation, proliferation, production and apoptosis (Bosman, 1993). They play pivotal roles in a variety of pathophysiological processes including wound healing (Waes, 1995; Zambruno et al, 1995) and are relevant to the project described in this thesis since TGF-β (Frank et al, 1996), IFN (Hertle et al, 1995) and NO (Igietseme, et al, 1996) have been observed to affect the functional activities of integrins.

Integrins are a family of integral membrane glycoproteins expressed by all cells including those major players in wound healing such as platelets, leukocytes, endothelia, epithelia, and mesenchymal cells (Waes, 1995). Like other cell surface glycoproteins, they serve as both adhesion and signal transduction molecules involved in a variety of cellular activities including embryonic development, the response of cells to mechanical stress, thrombus retraction, hemostasis, leukocyte homing and activation, bone resorption, tumor cell growth and metastasis, and programmed cell death (Juliano and Haskill, 1993; Juliano and Varner; 1993; Schwartz and Ingber, 1993; Wei et al, 1997). The integrin is a heterodimer of an α and a β subunit, non-covalently linked together. Currently, more than 22 integrins have been identified; they are made up from different combinations of 16 alpha and 8 beta subunits. The primary transcripts of the subunit genes undergo alternative splicing, adding additional complexity (Hynes, 1992). Integrins can bind both anchored ligands such as fibronectin, collagen or vitronectin in the ECM and soluble ligands such as fibrinogen in the plasma. They can also bind cell surface counter-receptors such as intercellular adhesion molecules on adjacent cells, leading to homo- or heterotypic aggregation. Binding of integrins to their ligands results in cross-linking or clustering of integrins on the cell surface, which are critical for the activation of responses inside the cells (Miyamoto et al, 1995). The engagement and clustering of integrin receptors leads to the formation of focal adhesions where integrins link to cytoskeletal complexes and bundles of actin filaments, activating a cascade of protein relocations and phosphorylations. These processes play pivotal roles in modulating cell adhesion and inducing the cell morphological changes involved in cell spreading and locomotion (Gumbiner, 1993; 1996; Pavalko and Otey, 1994). Although integrins do not have kinase activity, the focal adhesion complex contains several kinases and the Src homology (SH) domain containing adapter molecules (Clark and Brugge, 1995). The kinases involved in the focal adhesion complex include focal adhesion kinase, a tyrosine kinase that appears to play a central role in integrin-mediated signal transduction, and Src family of tyrosine kinases that contain an SH3 and SH2 domain (Clark and Brugge, 1995). Several other signal transduction pathways have also been implicated in integrin-mediated signaling. These include the Ras-MAP kinase pathway (Kapron-Bras et al, 1993), phospholipid kinases, phospholipases and protein kinase C pathways (Chen and Guan, 1994; Zhang et al, 1993). The involvement of multiple pathways in integrin-mediated signal transduction suggests the significance and complexity of this system.

2.4. NITRIC OXIDE: ACTIVE PARTICIPANT IN WOUND HEALING

2.4.1 Functional Activities of Nitric Oxide

Nitric oxide, originally identified as a blood vessel relaxing factor derived from endothelia (Palmer et al, 1987; 1988), has been shown to possess a variety of physiological and pathophysiological activities in several systems. In the central and peripheral nervous systems, it appears to be an inter- and intra-cellular messenger molecule, which functions as a neurotransmitter (Bredt et al, 1990). In the vascular system, NO plays a pivotal role in regulating blood flow by controlling blood vessel tone (Altman et al, 1994; Wei et al, 1994). In the immune system, all mononuclear leukocytes and neutrophils have the capacity, when stimulated, to synthesize NO, which is responsible for the cytotoxicity and inflammatory functions of macrophages (Cunha et al, 1993; Lorsbach et al, 1993) and neutrophils (Yui et al, 1991). NO also serves as a signaling molecule between the immune system and other organ systems by transmitting cytokine signals to the regulatory machinery of a variety of cells (Corbett et al, 1993; Schneemann et al, 1993).

2.4.2 Arginine-NOS Pathway: Biosynthesis of Nitric Oxide

The biosynthesis of NO is mediated by NOS, an NADPH-dependent enzyme that catalyzes the oxidation of one of the chemically equivalent guanidino nitrogen atoms of L-arginine with molecular oxygen to form NO and L-citrulline (Kwon et al, 1990; Leone et al, 1991). Since NO controls and regulates many critical processes in physiology and pathophysiology, and a variety of cell types synthesize NO, intensive interest has been developed and rapid progress has been made in this relatively new area of research. In parallel with our understanding of the biological actions of NO, important progress has been made in the understanding of the enzymology of NOS. Accumulating evidence shows that NOS is a complex enzyme involving several tightly bound redox cofactors (NADPH, FAD, FMN) that are clearly organized into distinct domains (Marletta, 1994). Another important co-factor is the reduced pterin that is tightly associated with NOS and can influence the enzyme activity (Rosenkranz-Weiss, 1994). Upon reduction and treatment with carbon monoxide, NOS shows a λ_{max} of ~ 450 nm, which is the relatively rare spectral characteristic of the large family of cytochrome P-450 enzymes (Nelson et al, 1993). Structurally, NOS is quite similar to NADPH cytochrome P-450 reductase since it contains a P-450-type heme moiety and the C-terminal part of the protein shows sequence homology to cytochrome P-450 reductase (Bredt et al, 1991; White et al, 1992). Functionally, NOS has been shown to carry out the P-450 electron transfer chemistry in formation of NO (Marletta, 1994).

Three isoforms of NOS are now distinguished by cloning of their genomic and complementary DNA in several mammalian species including humans (Bredt et al, 1991; Geller et al, 1993; Marsden et al, 1992; Nakane et al, 1993; Xie et al, 1992). Activities of two of these are dependent on elevated intracellular Ca^{2+} and extrinsic calmodulin and are constitutively expressed (cNOS). One seems to be restricted to endothelial cells (endothelial NOS, eNOS) while another is expressed in specific neurons (neural NOS, nNOS) of the central and peripheral nerve system and in skeletal muscles (Nakane et al, 1993). These two isoforms of NOS oxidize L-arginine to form small amounts of NO and citrulline in response to signals that elevate intracellular Ca^{2+}. This low level of NO serves as an intra-cellular messenger to exert important physiological functions such as maintaining the tone of blood vessels. The third isoform, inducible NOS (iNOS), is independent of elevated intracellular Ca^{2+} and possesses its own calmodulin in a tightly bound subunit (Cho et al, 1992). This isoform is not expressed under normal conditions, but can be induced in many types of cells by inflammatory cytokines and bacterial LPS. In the process of inflammation and immune responses, cytokines and bacterial products stimulate the expression of iNOS in a wide variety of cells that converts L-arginine to form high levels of NO, resulting in a diversity of beneficial and/or detrimental consequences (Petros et al, 1994; Weissman et al, 1992).

All of the NOS isoforms require NADPH and molecular oxygen as cosubstrates. The first NOS cDNA isolated was the neuronal cNOS isoform from rat cerebellum. Subsequently, cDNAs for cNOS from bovine aortic endothelial cells (Nishida et al, 1992) and human umbilical vein endothelial cells (Marsden et al, 1992) have been cloned and characterized. The cDNAs for iNOS from murine macrophages (Lyons et al, 1992; Xie et al, 1992) and human hepatocytes have also been cloned (Geller et al, 1993). The nNOS proteins have been purified from rat (Bredt et al, 1990; Schmidt et al, 1992) and porcine (Mayer et al, 1990) cerebellum and are shown to be dimeric cytosolic proteins with a molecular weight (MW) of 150 to 160 kDa. Another isoform of NOS from bovine aortic endothelial cells ~~has~~ been purified. It is a membrane-bound 135 kDa (Pollock et al, 1991). The iNOS protein has been purified from LPS/IFN γ-treated murine macrophage RAW 264.7 cells (Xie et al, 1992) and cytokine/ LPS-stimulated human hepatocytes (Geller et al, 1993). The iNOS activity has also been found in vascular endothelium and smooth muscle cells (Gross et al, 1991; Hiki et al, 1992; Radomski et al, 1990; Suschek et al, 1993). The iNOS isolated from murine macrophages and human hepatocytes is a dimer under native conditions with a molecular weight of 130 kDa. Macrophages also appear to have iNOS activity in the 100,000 *g* pellet suggesting that the macrophage iNOS also has the membrane bound subtype (Schmidt et al, 1992).

Analysis of NOS sequences shows that nNOS, eNOS and iNOS are distinct gene products, while a comparison of the nNOS sequences from rat and bovine brains indicates that they are derived from the same gene (Marletta, 1993). The N-terminus of eNOS, nNOS and iNOS show significant similarity (Lowenstein et al, 1992). All reported NOS's have highly conserved sequences for NADPH, FAD and FMN binding, and these sequences have homology to cytochrome P-450 reductase (Bredt et al, 1991). The loss of enzyme activity during the purification of nNOS from rat cerebellum led to the finding that calmodulin (CaM) was required for this NOS (Bredt and Snyder, 1990). Subsequently, eNOS was also shown to require Ca^{2+} and CaM. As expected, the sequences for neuronal and endothelial NOS's contain a consensus sequence for CaM recognition. This consensus sequence corresponds to residues 724-745 in nNOS (Lowenstein et al, 1992) and 493-512 in eNOS (Lamas et al, 1992). Interestingly, iNOS seems not to show a requirement for either Ca^{2+} or CaM, but it also has a CaM recognition sequence between residues 503 and 523 (Lowenstein et al, 1992; Lyons et al, 1992; Xie et al, 1992). In fact, the macrophage iNOS has a tightly bound CaM and requires very low concentrations of Ca^{2+} for activation, suggesting that iNOS may also be regulated by Ca^{2+} and CaM (Cho et al, 1992).

All the reported NOS cDNA's contain consensus sequences for phosphorylation, although the exact significance of phosphorylation in NOS is not clear (Bredt et al, 1991). The membrane-bound eNOS, but not the other isoforms, contains a myristoylation consensus sequence at the N-terminus. In addition, there are no sequences consistent with a transmembrane domain, suggesting that myristoylation is responsible for the membrane anchoring of this eNOS (Pollock et al, 1992).

The function of NOS is to catalyze the oxidation of L-arginine to NO and L-citrulline. This reaction is known to involve an initial hydroxylation of L-arginine to generate N^G-hydroxy-L-arginine. The heme moiety in NOS seems to be responsible for this step (White and Marletta, 1992; Pufahl and Marletta, 1993). It has been proposed that the heme moiety is also directly involved in the conversion of N^G-hydroxy-L-arginine to L-citrulline, although the chemical steps of the conversion are not clear (Marletta, 1993). The actual mechanism of the conversion of N^G-hydroxy-L-arginine to L-citrulline is still not known, as Marletta (1994) stated that: "This proposed mechanism involves a heme ferric peroxide ($Fe(III)$-OO^-) nucleophile as a key reaction intermediate. In addition, the mechanism proposes that N^G-hydroxy-L-arginine reduces the ferrous oxy-heme complex ($Fe(II)$-O_2) to form $Fe(III)$-OO^- generating a radical species on the substrate that would ultimately become the odd electron radical on NO". The proposed mechanism remains to be confirmed by experimental procedures.

Although the first evidence suggesting that mammals produced nitrogen oxides appeared as early as 1916 (Mitchell et al, 1916), synthesis of nitrogen oxides by specific mammalian cells was only conclusively demonstrated more recently by Stuehr and Marletta (1985) when they found that murine macrophages synthesized nitrite and nitrate upon stimulation with bacterial LPS. Subsequently, L-arginine was found to be the substrate for murine macrophage nitrite synthesis and the conversion of L-arginine to nitrite was required for macrophage-induced cytotoxicity (Hibbs et al, 1987).

The synthesis of NO in rodent cells has been well studied. In addition to NO production in macrophages, neurons and endothelial cells, many other types of rodent cells including smooth muscle cells, bone marrow cells, mesangial cells, cardiac myocytes, hepatocytes, astrocytes, fibroblasts, keratinocytes, neutrophils, lymphocytes, and several tumor cells lines, are capable of NO synthesis (Nussler et al, 1993; Taylor-Robinson et al, 1994). In humans, as in other mammals, most types of cells have been shown to synthesize NO (Nussler et al, 1993). However, NO production by human cells is much lower than that by rodent cells and there is some controversy whether or not human monocytes/macrophages produce NO (Denis, 1994). The physiological effects of low levels of NO produced by eNOS on homeostasis are well characterized in all species, but the physiological and pathophysiological effects of high concentrations of NO produced by iNOS on host defense have only been studied in rodents. Rodent macrophages produce large amounts of NO upon stimulation with pro-inflammatory cytokine, while the macrophages from other species produce relatively small amounts of NO (Moncada et al, 1991).

2.4.3 Regulation of Nitric Oxide Biosynthesis

The regulation of NOS expression and NO production presents a very complex system, involving multiple controls at the transcriptional, translational, and post-translational levels. Different regulatory mechanisms seem to exist for eNOS and iNOS (Nathan and Xie, 1994). For eNOS, the expression is regulated by various physical and humoral stimuli. The factors that up-regulate eNOS expression and/or activity include chronic shear stress exerted by flowing blood on endothelia (Inoue et al, 1995), bacterial endotoxin (Greenberg et al, 1995), and IL-1 (MacNaul and Hutchinson, 1993). The factors that down-regulate eNOS expression include TGF-β and TNF (Yoshizumi et al, 1993). Cytokines including

IL-1, TNF and IFN-γ, and bacterial LPS stimulate the expression of iNOS while several endogenous factors such as corticosteroids (Di Rosa et al, 1990), retinoids (Hirokawa et al, 1994), trace amounts of LPS (Bogdan et al, 1993), NO itself (Griscavage et al, 1993), and cytokines including IL-4 (Bogdan et al, 1994), IL-10 (Gazzinelli et al, 1992), FGF (Goureau et al, 1993) and TGF-β (Gazzinelli et al, 1992) have inhibitory effects.

2.4.4 The Role of Nitric Oxide in Wound Healing

It is well-known that arginine is an essential amino acid for the injured animal (Seifter et al, 1978) and it stimulates wound healing in humans (Barbul et al, 1983). Arginine has been observed to have thymotropic effects and to stimulate lymphocyte immune responses (Barbul et al, 1990; Kirk et al, 1993a). The mechanisms of action of arginine in enhancement of wound healing were only revealed recently when the arginine-NOS pathway was found (Kirk et al, 1993b). It is now clear that NO, the metabolite of arginine in this pathway plays a major role in wound healing, directly through its effects on wound collagen synthesis and deposition (Kirk et al, 1993b; Owens et al, 1996; Schäffer et al, 1996). NO may also participate in regulation of wound healing through several indirect mechanisms including vasodilatation, inhibition of platelet aggregation, its anti-proliferative, and anti-bacterial effects, induction of apoptosis in macrophages (Albina et al, 1993; Sarih et al, 1993) and inhibition of apoptosis in lymphocytes (Mannick et al, 1994).

The role of NO in wound healing, as in other physiological and pathological processes, is a matter of quantity. Platelet aggregation is an essential process of platelet activation and NO has been observed to inhibit this process

(Mollace et al, 1991; Salvemini et al, 1989). This effect of NO on platelet aggregation is demonstrated by the observation that activated human mononuclear cells inhibit platelet aggregation. This effect is potentiated by superoxide dismutase, which reduces levels of the NO scavenger superoxide, and is inhibited by oxyhemoglobin, a very potent NO scavenger. The inhibitory effect of mononuclear cells on platelet aggregation was prevented by their pre-incubation with the NOS inhibitor N^G-monomethyl-L-arginine methyl ester but is not affected by pre-incubating the cells with catalase or mannitol (Salvemini et al, 1989). It is well known that platelet aggregation is necessary for platelet activation and granule release. Thus, inhibition of platelet aggregation by NO may potentially inhibit platelet activation and cytokine release.

Fibroblasts have been shown to produce NO upon stimulation with cytokines and LPS (Jorens et al, 1992; Willis et al, 1994), suggesting that NO produced by fibroblasts may play a role in regulating the process of inflammation and wound healing through its vasodilatation and antiproliferative effects (Jorens et al, 1992). It has been observed that NO is a strong inducer of apoptosis for macrophages, cells that are well known to play major roles in wound healing (Albina et al, 1993). This suggests a negative feedback mechanism for NO in regulation of the inflammatory response during the acute stage of wound healing, in which chemo-attractants released by activated platelets and bacterial contamination induce macrophage infiltration and cytokine production. The proinflammatory cytokines such as IL-1, TNFs, and IFN-γ and bacterial products such as LPS induce a variety of cells including macrophages in the wound to produce NO, which in turn, causes apoptosis of macrophages resulting in reduced cytokine and NO production in the wounds (Albina et al, 1993). Furthermore, NO may affect the wound healing process by inhibiting keratinocyte proliferation (Benrath et al, 1995; Heck et al, 1992). This inhibitory effect is antagonized by epidermal growth factor (EGF), which has been shown to inhibit the enzymatic activity of NOS (Heck et al, 1992).

2.4.5 The Role of Nitric Oxide in Hypertrophic Scar Formation: Nitric Oxide and Fibrogenic Cytokines

Several lines of evidence indicate that NO may play a role in HSc formation. Firstly, it has been observed that TGF-β down-regulates pro-inflammatory cytokine-induced expression of iNOS and NO production in several types of cells including mouse macrophages and retinal pigment epithelial cells (Vodovotz et al, 1993; 1996), rat pulmonary artery smooth muscle cells (Finder et al, 1995; Perrella et al, 1996), human keratinocytes (Arany et al, 1996), bovine endothelial cells (Inoue et al, 1995), and C2C12 skeletal myocytes (Williams et al, 1996). This inhibitory effects of TGF-β on NO production and NOS expression is further demonstrated in TGF-β1 null mice, in which iNOS expression and NO production is dramatically increased (Vodovotz et al, 1996) along with massive auto-inflammatory reaction of multiple organ systems of the animal. It is well-known that NO may act as a negative regulator for several biological process including inflammatory responses and cytokine production during tissue injury or infection. Lack of this negative feedback loop may lead to over-response in inflammation, resulting in over-production of fibrogenic cytokines. It is also known that NO has anti-proliferative effects on a variety of cells including fibroblasts. Low levels of NO induced by TGF-β may lead to over-proliferation of fibroblasts, resulting in HSc formation. Secondly, NO and its oxidative metabolite peroxynitrite has been reported to activate collagenase and stromelysin, two metalloproteinases that are important during inflammation and wound healing (Murrell et al, 1995). This is demonstrated by increased collagenase and stromelysin activity in bovine and human cartilage chondrocytes and explants after IL-1β and LPS treatment. The metalloproteinase activity is induced by the NO donor, S-nitroso-N-acetyl-D, L-penicillamine and inhibited by the NOS inhibitor, N^G-nitro-L-arginine methyl ester (Murrell et al, 1995; Okamoto et al, 1997; Trachtman et al, 1996). Recently, NO has been reported to activate a 72-kDa neutral matrix metalloproteinase in mesangial cell cultures *in vitro* (Trachtman et al, 1996). These results suggest that NO plays a regulatory role in metalloproteinase activation in articular chondrocytes and mesangial cells. It is possible that NO also regulates collagenase activity in fibroblasts and skin.

Recent research described in this thesis demonstrated that HSc fibroblasts and tissue produced significantly less NO and expressed less eNOS proteins than their counterpart normal cells and tissues. The lower levels of endogenous NO in fibroblasts may enhance proliferation of these cells and hinder the activation of collagenases, resulting in excess collagen production and deposition. These results indicate that NO may be responsible, in part, for HSc formation (Chapter Four).

2.5. TGF-β: CONSEQUENTIAL FIBROGENIC CYTOKINES

2.5.1 Discovery and Characteristics

Transforming growth factors were originally identified based on their ability to induce reversible phenotypic transformation of normal fibroblastic indicator cells *in vitro* (Roberts and Sporn, 1985). Two distinct classes of TGFs, TGF-α and -β, have been purified to homogeneity. They are distinguished both chemically by their unique amino acid sequences and biologically by their different activities on cells. Type alpha TGFs have strong homology to epidermal growth factor and are composed of single peptide chains about 50-53 amino acids cross-linked by three disulfide bonds. Type beta TGFs have a homodimeric structure comprised of two chains of 112 amino acids, each containing nine cysteine residues. They belong to a superfamily of signaling peptides that include TGF-β family, the developmental regulators activin and inhibin, Müllerian inhibiting substance, *Xenopus* Vg-1, the *decapentaplegic* gene product in *Drosophila*, and the bone morphogenic proteins. The molecules of this superfamily share between 23% and 40% sequence identity in their C-terminal portions, but they do not display any sequence homology to any other known growth factors.

At least 5 members of the TGF-β family, TGF-β1 to β5, have been identified in the animal kingdom. Three of them, TGF-β1, -β2 and -β3 have been found in mammals. These three mammalian TGF-β molecules share 70% to 80% sequence identity, and the amino acid sequences of the mature, proteolytically processed forms of each member is almost entirely conserved among species (Lawrence, 1996). Each isoform is encoded by a unique gene on different chromosomes. All three isoforms are synthesized and secreted by most cell types, generally in a precursor form with 390-412 amino acids, requiring activation before they can exert biological activity. The biologically active form of TGF-β occurs as a dimer linked by a single disulfide bond at Cys 77 of both 112 amino acid monomers and a molecular mass of 25 kDa. It is demonstrated that the 112 amino acid mature form of TGF-β is the proteolytic product of the 390 amino acid precursor.

Chemically, TGF-β is very acid resistant. This property makes it easy to isolate and purify this molecule from tissue or cell cultures by acid ethanol extraction. It is now well established that type beta TGFs are multifunctional peptides that play fundamentally regulatory roles in development, osteogenesis, wound healing, immuno-modulation and pathogenesis of diseases.

2.5.2 Biological Activities of TGF-β

The biological activities of type beta TGFs are diverse. Their actions on target cells are dependent on a number of parameters, which include the origin and state of differentiation of the cells, local concentrations of various activating and inhibiting molecules, the presence of other growth factors and the architecture of the ECM. Generally, they inhibit the proliferation and function of epithelial cells, endothelial cells, lymphoid and myeloid cells while stimulating the proliferation and activities of mesenchymal cells. The mechanisms of these different effects are not clear but it can be speculated that they may result from differential expression of receptors for TGF-β and different signal transduction pathways.

Accumulating evidence indicates that type beta TGFs are involved in embryogenesis (Akhurst et al, 1990; Bonyadi et al, 1997) and bone formation (Rosen et al, 1988). In the developing mouse embryo, different types of TGF-β are expressed by different types of cells at certain times suggesting that the isoforms of TGF-β may differentially regulate cell proliferation and differentiation, which is absolutely required in organogenesis (Millan et al, 1991; Roberts and Sporn, 1992). Type beta TGFs play a major role in wound healing and fibroproliferative disorders. Early work demonstrated that TGF-β accelerates wound closure and increases breaking strength in experimental animals (Sporn et al, 1983).

Consequently, TGF-β has been shown to stimulate fibroblast collagen, fibronectin, proteoglycan and GAG synthesis, to enhance neovascularization, and to modulate production of a variety of proteases and their inhibitors, ultimately resulting in the accumulation of matrix (Sporn et al, 1987). Interestingly, different isoforms of type beta TGFs appear to have different effects on wound healing. All of them enhance wound healing by stimulating fibroblast proliferation and ECM production. TGF-β1 and TGF-β2 seem to be equipotent in eliciting these effects, while TGF-3 is less fibrogenic and has been reported to reduce scar formation (Shah et al, 1995). It is well-known that TGF-β increases production of type I, III, and V collagens by normal fibroblasts and fibroblasts derived from a variety of fibroproliferative disorders (Ignotz et al, 1987; Lawrence, 1996). Recently, TGF-β has been shown to stimulate adhesion and integrin expression by mesangial cells, suggesting that TGF-β may also regulate cell activities through modulating adhesion receptors, which are well-know to exert a variety of physiological functions on cells (Kagami et al, 1996).

2.5.3 TGF-β Genes and Transcription

It has been shown that the genes for type beta TGFs are well-conserved among mammalian species. The TGF-β genes from different sources have very high sequence homology to the human TGF-β isoforms. In humans, the genes for the various isoforms are found on different chromosomes and encode unique transcripts. The TGF-β1, -β2, and β3 genes in humans are located at 19q13, 1q41, and 14q24, respectively (Barton et al, 1988; Fujii et al, 1986; ten Dijke et al; 1988). The gene for the TGF-β2 is transcribed as 4.1 kb, 5.1 kb and 6.5 kb molecules that are considered to be processing intermediates of the same primary mRNA transcript. The TGF-β1 and -β3 genes encode a 2.5 kb and a 3.0 kb transcript, respectively.

Based on genomic sequencing, cDNA cloning and S1 nuclease mapping, the gene for human TGF-β1 has been estimated to be more than 100 kb long and composed of 7 exons and 6 introns. The position of the splice junctions along the gene are highly conserved, being identical to those in both bovine and porcine TGF-β1. The gene structure for TGF-β2 and TGF-β3 isoforms is similar suggesting that the TGF-β isoforms may have arisen by duplication of a single ancestral DNA sequence. The coding sequence corresponding to the 390 amino acid human TGF-β1 precursor and the 5'-flanking regions of the gene have been mapped (Derynck et al, 1987). The TGF-β1 promoter sequence extends 1400 bp upstream of the transcription start site. This region is G-C rich and contains two promoter regions, two negative regulatory regions and one region with enhancer-like activity (Kim et al, 1989a). The promoter regions contain several binding sites for putative transcription factors including NF-1, SP-1, AP-1 and AP-2 (Geiser et al, 1993; Kim et al, 1990). The negative regulatory regions contain fat-specific element 2 and a putative IFN responsive element, which provides the structural evidence for IFN regulation of TGF-β expression (Kim et a, 1989a).

Experimental evidence indicates that the expression of TGF-β1 is mainly regulated by the AP-1 complex although other nuclear factors may also regulate TGF-β1 expression. The precise mechanism(s) of regulation is not yet clear. It is well-known that AP-1 expression is induced by a variety of factors indicating the complexity of the regulation of TGF-β1 expression (Kim et al, 1990). It has been observed that TGF-β1 increases its own expression in normal and transformed cells (Obberghen-Schilling et al, 1988) and the auto-induction is mediated by AP-

1 binding sequences in the promoter region (Kim et al, 1989b). The autocrine regulation of TGF-β1 expression may serve to amplify an initial signal. This process may play an important role in directing wound healing and regulating the immune response.

2.5.4 TGF-β Protein Structure and Activation

Type beta TGFs are synthesized and secreted by a variety of transformed and non-transformed cells in a latent form that is not able to bind to its cell surface receptors and cannot induce cellular responses (Lawrence et al, 1985). Thus, they are biologically inactive. It is assumed that the receptor binding and signal initiating sequences are masked by a peptide called latency associated peptide (LAP), which is part of the originally translated TGF-β precursor peptide (Derynck et al, 1985; Lyons et al, 1988; Wakefield et al, 1988). The LAP consists of amino acids 1 to 278 from the N-terminal of the pre-pro-portion minus the 23 amino acid signal sequence (Derynck et al, 1985; Tsuji et al, 1990). Biochemical analysis of the LAP-TGF-β complex shows that the LAP is glycosylated on asparagine residues by mannose 6-phosphate chains, which are lysosomal sorting markers and can be recognized extracellularly by the IGF-II receptor. It is assumed that mannose 6-phosphate side-chains may serve to target the TGF-β precursor for extracellular activation (Dennis and Rifkin, 1991; Derynck et al, 1985; Tsuji et al, 1990).

The latent TGF-β can be activated *in vitro* by physical or chemical manipulations such as heating to 100°C for 3 minutes, incubating with 8M urea, acidification and alkalinization (Lawrence et al, 1985). However, these conditions are unlikely to be the mechanisms involved in the activation of type beta TGFs *in* *vivo*. Disruption of the latent complex, either by conformational changes induced by another binding component, or by complete dissociation of the LAP by proteolysis, results in the activation of TGF-β. It is assumed that enzymatic cleavage, binding of bioactive proteins that induce conformational change, or mild acidic microenvironment under certain conditions, are likely to be the mechanisms operating *in vivo*. These assumptions have been demonstrated by experiments using radioreceptor assay and pericyte/endothelium co-culture systems (Lyons et al, 1988; Orlidge et al, 1987).

Plasmin, a broad spectrum protease usually associated with the cell surface, has been intensively studied for its ability to activate type beta TGFs. It has been reported that plasmin can activate the latent TGF-β produced by chicken embryo and mouse fibroblasts. It was demonstrated that fibroblast-conditioned medium treated with plasmin showed TGF-β activity similar to that of mild acid-treated-conditioned medium (Lyons et al, 1988). In the pericyte/endothelium co-culture system, plasmin inhibitors such as α2-plasmin inhibitor eliminated the growth arrest effect on pericytes and smooth muscle cells, which is considered to be induced by activated TGF-β (Antonelli-Orlidge et al, 1989; Sato and Rifkin, 1989). Enzymatic removal of polysaccharide side-chains of latent TGF-β also results in activation. It has been reported that treatment of human platelet-derived TGF-β with endoglycosidase F, which removes the N-linked carbohydrate complex, leads to its activation. Moreover, incubation of latent TGF-β with sialidase also leads to its activation, suggesting that the sialic acid side-chains in the precursor are involved in the latency of type beta TGFs (Miyazono and Heldin, 1988; Miyazono et al, 1989).

2.5.5 TGF-β Receptors and Regulatory Binding Molecules

A total of six receptors, designated TGF-β receptor types I to VI, have been identified. Receptor types I - III are most widely distributed and are found on most cells with a few exceptions that include human retinoblastoma, rat phaeochromocytoma and undifferentiated embryonic carcinoma cells (Massagué et al, 1985). The functions and distribution of type IV, V and VI receptors for TGF-β are not well-defined but their molecular weights are approximately 60 kDa, 400 kDa and 180 kDa respectively. Radioreceptor binding studies show that type beta TGFs have high affinities for these receptors with dissociation constants of 5-25 picomolar for the types I and II receptors and 200 picomolar for type III (Massagué et al, 1985).

Types I and II receptors of TGF-β are glycoproteins with molecular masses of 50-80 kDa and 85-140 kDa, respectively. They contain N-linked carbohydrate complexes that are not essential for expression or ligand binding but may play roles in stability or affinity (Wakefield et al, 1987). They are specific for type beta TGFs since other growth factors such as EGF, PDGF, IGF-1, IGF-2 and TGF-α cannot bind (Boyd et al, 1989; Frolik et al, 1984). It has been demonstrated that type I and type II receptors may interact with each other as a receptor complex and are the active mediators of the TGF-β responses in most cells because mutation or deletion of one of them abolishes the response (Boyd et al, 1989; Laiho et al, 1990).

The type III receptor for TGF-β, also known as betaglycan, is a proteoglycan with a 110-140 kDa core protein that contains a high affinity binding site for TGF-β (Andres et al, 1989; 1991). The GAG chains, composed of chondroitin sulfate and heparan sulfate, have little effect on TGF-β binding or TGF-β induced biological activities (Segarini and Seyedin, 1988). Both integral membrane and soluble forms of betaglycan have been identified. The biological significance of the soluble form of betaglycan is not yet known. Although the integral membrane form of the type III receptor contains a 41 amino acid cytoplasmic domain with a putative phosphorylation site for protein kinase C, it is not involved in signal transduction (Andres et al, 1991). The biological role for this type receptor is assumed to be presenting TGF-β to the signaling receptors or activation systems and to served as a reservoir or clearance system for the active form of TGF-β (Massagué et al, 1990).

Other TGF-β binding molecules include α2-macroglobulin, decorin, endoglin, and thrombospondin. The natural endopeptidase inhibitor α2-macroglobulin is a circulating protein that undergoes a rapid conformational change, when a susceptible peptide bond on its surface is cleaved, causing it to bind and sterically inhibit the enzymatic activity of the proteinase (Tsuji et al, 1990). It has been reported that serum α2-macroglobulin often forms covalent and non-covalent associations with TGF-β, which is inactive although the LAP is not found in the complex. Interestingly, latent TGF-β with LAP does not bind α2-macroglobulin, but upon activation by transient acidification it gains this capability (Tsuji et al, 1990). These findings suggest a possible role for α2-macroglobulin as a regulatory scavenging molecule that binds and neutralizes the biological activity of TGF-β (Phan et al, 1989; Tsuji et al, 1990).

Decorin, a small interstitial proteoglycan containing dermatan sulfate /chondroitin sulfate with a globular core protein, is thought to associate with collagen in the extracellular matrices of many tissues (Kresse et al, 1994; Schonherr et al, 1995). It has been reported that decorin forms a complex with TGF-β and inhibits its biological activity in mink lung epithelial cell bioassays. This inhibitory effect may due to association of TGF-β with decorin affecting its receptor binding ability (Yamaguchi et al, 1990). However, the formation of the decorin-TGF-β complex only abolishes certain TGF-β-mediated activities such as the up-regulation of biglycan synthesis in osteosarcoma cells while some activities such as down-regulation of proteoglycan-100 and stimulation of fibroblast populated collagen lattices are not affected (Hausser et al, 1994). The biological significance of these differences in decorin-mediated TGF-β regulation is not yet known.

Thrombospondin, a multi-domain glycoprotein found in the α-granules of platelets, wound fluid, and embryonic tissues, has been shown to bind and activate latent TGF-β without removing its LAP (Schultz-Cherry et al, 1994). These observations provide evidence that conformational changes in the latency complex may unmask cryptic structures and activate the cytokine.

2.5.6 TGF-β Signal Transduction

Although several intracellular pathways for TGF-β-mediated signal transduction have been proposed, the direct transduction cascade initiated by the TGF-β receptor system is not known at present. It is now established that the type I and type II receptor-mediated signal transduction is initiated at the cytoplasmic domains of the receptors, which are auto-phosphorylated at serine/threonine residues immediately after ligand binding (Massagué and Weis-Garcia, 1996). Recently, the receptors have been reported to be autophosphorylated at tyrosine residues as well, suggesting that types I and II receptors of TGF-β use not only serine/threonine kinase, but also tyrosine kinase (Lawler et al, 1997). The tyrosine kinase activity of the receptors provides evidence for the involvement of the Src family of tyrosine kinases in TGF-β signal transduction (Atfi et al, 1994). Down-stream of the kinases of the receptor complexes, several signaling pathways have been identified. One of the recently elucidated pathways is the mitogen-activated protein kinase (MAPK) pathway, in which MAPK kinase kinase, also know as TAK1 (MAPKKK), is activated after receptor occupancy (Shibuya et al, 1996; Yamaguchi et al, 1995). This pathway leads to a diversity of cellular activities including proliferation, differentiation, secretion, growth arrest and death, depending on the down-stream molecules involved. The cyclin E/cyclin-dependent kinase (cdk) pathway is another one in which type beta TGFs are involved (Schoecklmann et al, 1997). One group of down-stream molecules in this pathway is the cell cycle regulatory retinoblastoma proteins (Schoecklmann et al, 1997), which are differentially regulated by TGF-β in different types of cells (Ravitz and Wenner, 1997). Several receptor interacting proteins and potential mediators, including farnesyl transferase-α, Mothers against decapentaplegic (Mad)-related proteins, and TAB1 and TAB2 (TAK1 binding protein 1 and 2) that activate MAPKKK, have been suggested to participate in signal transduction by TGF-β receptors. Recent advances, particularly in our understanding of the function of Mad related proteins, are providing new insights into how the TGF-β superfamily exerts its diverse biological activities (Zhang et al, 1996).

2.6 THE INTERFERONS: ANTI-FIBROPROLIFERATIVE AGENTS WITH THERAPEUTIC POTENTIAL

2.6.1 Discovery and Classification

Originally identified and named for their actions as antiviral agents produced by viral infected cells, IFNs are now recognized to be a family of multifunctional cytokines that are involved in many aspects of biological activity (Farrar and Schreiber, 1993; Larner and Reich, 1996). Two distinct classes of interferons have been characterized based on their sequence identity and their receptor interactions (Diaz and Testa, 1996; Farrar and Schreiber, 1993; Larner and Reich, 1996). Type I interferons, which include IFN-α and -β, are structurally and genetically related proteins that interact with a common receptor. They are constitutively expressed at low levels by all nucleated cells, and undergo rapid and transient induction following viral infection (Farrar and Schreiber, 1993; Pelligrini and Schindler, 1993). Type II interferon, also known as IFN-γ or immune IFN, is synthesized and secreted only by activated T-lymphocytes and natural killer cells and interacts with a distinct cell surface receptor. The receptors for type I and type II interferons use distinct pathways for signal transduction but share some intracellular receptor-associated molecules, which are also shared by other cytokine receptors, for signaling (Farrar and Schreiber, 1993; Muller et al, 1994). The multifunctional nature and biological potential of IFN family has led to their use in the clinical management of numerous pathological conditions including viral infections, hematopoietic malignancies and fibroproliferative disorders.

2.6.2 Interferon Genes and Protein Structures

The gene structure for the human IFN family has been characterized. The genes for IFN-α and -β are located on chromosome 9 and contain no introns (Diaz and Testa, 1996). More than 12 genes have been identified for IFN-α. These genes code structurally different proteins that are divided into 2 classes, IFN-α1 and IFN-α2. The genes for IFN-α1 encode mature proteins of 165 or 166 amino acids, while the mature IFN-α2 proteins consists of 172 amino acids. The IFN-α proteins are synthesized as pre-interferons, containing a 23 amino acid leader sequence that is cleaved during maturation (Diaz and Testa, 1996). The IFN-α molecules contain four highly conserved cysteine residues which form two disulfide bridges between Cys 1 to 99 and Cys 29 to 139. The second disulfide bridge is essential for the biological activity of IFN-α (Wetzel, 1981).

A single gene has been identified for IFN-β which is commonly known as "fibroblast interferon". This gene shares approximately 45% sequence identity with the IFN-α genes and codes for a 166 amino acid mature protein (Derynck et al, 1980). It contains a promoter sequence called the interferon gene regulatory element (IRE), located -77 and -37 from the transcription starting site. This sequence is required for maximal induction of the gene and bears strong nucleotide sequence identity to the inducibility sequence of the IFN-β genes (Goodbourn et al, 1985).

The gene for human IFN-γ is approximately 6 kb in length and located on the long arm of chromosome 12. It contains four exons, which code for 38, 23, 61 and 44 amino acids of the mature protein, and three introns (Naylor et al, 1983). The 5' flanking region contains several regulatory sequences that are responsible for the induction and transcriptional regulation of this cytokine (Hardy et al, 1985; 1987). The four exons of human IFN-γ are transcribed to a 1.2 kb mRNA, which is translated into a 166 amino acid precursor with a 23 amino acid signal sequence (De Maeyer and De Maeyer-Guignard, 1992). Cleavage of this signal sequence yields a 143 amino acid mature protein with a molecular weight of 17 kDa (Derynck et al, 1982). The molecules of mature human IFN-γ contain two N-glycosylation sites at positions 25 and 97. The modification of glycosylation does not appear to affect the functional activities of this molecule, however, it may make IFN-γ more resistant to proteolytic cleavage and thus increase its biological half-life. The biologically active form of IFN-γ is a homodimer of two non-covalently associated polypeptides (Scahill et al, 1983; Walter et al, 1995).

2.6.3 Interferon Receptors

Two types of receptor have been characterized for human IFNs based on their ligand binding and signal transduction properties (Aguet, 1991). The type I receptor, which is shared by IFN-α and -β, has been found to be a disulfide-linked dimer consisting of two subunits, designated α and β or IFNAR-1 and IFNAR-2. The α subunit or IFNAR-1 is a glycoprotein with a molecular mass of 110 kDa. The β subunit or IFNAR-2 occurs as two forms, a long form with molecular weight of 95 to 100 kDa and a short form of 55 kDa (Domanski et al, 1995). It has been demonstrated that IFNAR-2 plays a major role in affinity determination and differential recognition of type I IFNs, while IFNAR-1 modulates both the ligand affinity and selectivity of the IFNAR-1/IFNAR-2 receptor complex (Cutrone and Langer, 1997). The gene for the type I IFN receptor has been mapped to 3 distinct loci, which code for different subtypes of the receptor, on human chromosome 21 (Raz et al, 1995).

The gene for the type II IFN receptor has been localized to chromosome 6, and contains six exons that result in a 2.3 kb transcript. The gene product is an 472 amino acid mature protein which is glycosylated, to give an 80 kDa single-chain glycoprotein receptor (Hershey and Schreiber, 1989). This receptor is unable to confer biological activity to IFN-γ unless complexed to a species-specific transmembrane glycoprotein encoded on chromosome 21, which does not contribute to the extracellular binding of IFN-γ (Soh et al, 1994). Upon binding of IFN-γ to the receptor, specific serine and threonine residues in the receptor become phosphorylated. Phosphorylation of these residues initiates signal transduction (Hershey et al, 1990).

2.6.4 Signal Transduction

Type I and type II IFNs use distinct signal transduction pathways to exert their biological functions. In the type I IFN receptor-mediated signaling, the α subunit of the type I receptor is physically associated with a cytoplasmic tyrosine kinase Janus kinase 1 (JAK1) that is directly involved in signal transduction. Binding of type I IFN to the β subunit of the transmembrane receptor stimulates the activation of the α subunit associated tyrosine kinases of the JAK family. Subsequently, latent cytoplasmic transcription factors such as signal transducers and activators of transcription (STATs) are activated by tyrosine phosphorylation (Larner and Reich, 1996). The phosphorylated STAT proteins, which include p84/p91 and p113, combine with another cytoplasmic 48 kDa protein to form the IFN-α-stimulated gene factor 3 complex (ISGF-3) which rapidly translocates to the nucleus and binds to cis-acting elements on interferon-stimulated gene elements (ISGE) to initiate their transcription (Schindler et al, 1992).

The type II receptor mediates similar processes for IFN-γ signal transduction. The binding of type II IFN to its receptor causes the recruitment and activation of JAK1, and subsequent phosphorylation of STAT1 at Tyr701. The phosphorylated STAT1 is thought to translocate to the nucleus where it oligomerizes to form the gamma-interferon activated fragment or GAF, which then binds and transactivates genes containing the gamma-interferon activated sequences or GAS (Larner and Reich, 1996).

The genes of a variety of molecules contain ISGE and GAS, which provide the molecular basis for IFN regulation of these genes. It has been shown that the gene for mouse iNOS contains the binding site for ISGE at the 5'-flanking region. The location for this site is -913 to -1029 upstream of the transcription start site. In conjunction with another site that contains LPS-related responsive elements, including a binding site for nuclear factor IL-6 and NF-κB, the ISGE binding region induces 750-fold increase in luciferase activity over the minimal promoter construct (Lowenstein et al, 1993).

2.6.5 Anti-fibrogenic Effects of Interferons

Besides their antiviral and immunoregulatory effects, IFNs have been shown to be anti-fibrotic agents. A number of *in vitro* studies have demonstrated that type I and type II IFNs are able to reduce the synthesis of collagen and inhibit the proliferation of normal human fibroblasts (Clark et al, 1989; Jimenez et al, 1984) and fibroblasts derived from fibroproliferative disorders (Berman and Duncan, 1989; Duncan and Berman, 1987). IFN-γ has been reported to reduce collagen synthesis by decreasing type I and type III collagen mRNA levels but does not affect the transcriptional rates, suggesting that regulation may occur at the level of transcript stability (Czaja et al, 1987). It has been shown that IFN-γ and IFN-α2b reduce type I collagen mRNA expression and have anti-proliferative effects on normal and hypertrophic scar fibroblasts (Harrop et al, 1995). Although the mechanisms of inhibition of collagen synthesis are not fully understood, IFN-α2b has been observed to act at a number of different levels: decreasing type I collagen mRNA expression, reducing post-translational hydroxylation of collagen, and stimulating the intracellular degradation of newly synthesized collagen (Tredget et al, 1993). In addition, type I and type II IFNs have also been shown to enhance the synthesis of the metalloproteinases collagenase and stromelysin by fibroblasts (Duncan and Berman, 1989; Tamai et al, 1995). This induction is mediated by nuclear factor AP-1 (Sciavolino et al, 1994). It has been speculated that the anti-fibrogenic effects of IFNs are mediated by inhibiting collagen synthesis, enhancing metalloproteinase production and antagonizing fibrogenic effects of TGF-β (Gurujeyalakshmi and Giri, 1995).

2.6.6 Therapeutic Application of Interferons

Clinically, both type I and type II IFNs have been employed for treatment of a variety of human diseases such as hematological diseases including acute lymphoblastic leukemia (Ochs et al, 1991) and chronic myelocytic leukemia (Dow et al, 1991) in pediatric patients and fibroproliferative disorders including hepatic cirrhosis, systemic sclerosis and rheumatoid arthritis (Castilla et al, 1991; Hein et al, 1992; Veys et al, 1988). Intralesional injections of IFN-α2b and -γ have been used to treat keloids leading to gradual reduction of the lesion and decreased collagen production by the fibroblasts derived from the lesion after treatment (Granstein et al, 1990; Larrabee et al, 1990). Subcutaneously administered IFN-α has also been used for the treatment of Type B and C hepatitis, in which patients suffering from chronic hepatitis demonstrated approximately a 50% response rate with good tolerance. The responses of hepatitis patients to IFN-α treatment was demonstrated by normalization of serum procollagen type III peptide, liver enzyme function, and TGF-β1 mRNA in liver tissues at the end of the therapy (Castilla et al, 1991). It has been proposed that the endogenous IFNs are important in development of those fibroproliferative disorders in that low levels of IFN-α and -γ were found in patients with alcoholic cirrhosis and keloids, in which the patients may have a systemic immune defect including a deficiency of endogenous IFN production as contributing factors to the fibroproliferative disorders (McCauley et al, 1992; Vicente-Gutierrez et al, 1991).

2.7 SUMMARY

Wound healing is a strictly regulated process involving both cellular and ECM components leading to reconstitution of the integrity and physiological functions of damaged tissues following injury. Cytokines and other molecules produced by platelets, leukocytes, endothelia, epithelia, and mesenchymal cells play major role in regulating this process. Malfunction of one or more of these molecules may result in a non-healing wound or HSc formation. Among these molecules, NO, type beta TGFs and IFNs are three major players in regulation of wound healing and HSc formation. They represent three individual components of a complex network of regulation and are mutually regulated among themselves. Furthermore, they function synergistically or antagonistically in the process of wound healing to maintain the balance of ECM metabolism. Although a huge amount of information is now available concerning the individual activities of these molecules, we do not yet have a complete understanding of the interactions of these three molecules. Better understanding of their interactions and functional activities would be useful for providing guidance in the clinical management of wound healing and HSc.

2.8 THESIS AIMS

Based on the literature reviewed above, I hypothesize that human dermal fibroblasts possess NOS and produce NO, that the NOS expression and NO production is altered in HSc fibroblasts due to phenotypic alteration, that the HSc tissues and fibroblasts produce more TGF-β mRNA and protein, and that treatment with IFN increases NO synthesis but inhibits TGF-β production. To test these hypotheses, two series of experiments, one dealing with NOS in human dermal fibroblasts and the other dealing with TGF-β in HSc tissues and fibroblasts after IFN treatment, were carried out.

Specific aims of the thesis:

1. To determine whether human dermal fibroblasts, like mouse and rat fibroblasts, express NOS and produce NO *in vitro* (Chapter Three).
2. To determine the isoforms of NOS used by human dermal fibroblasts to produce NO and the metabolic pathway for NO production (Chapter Three).

3. To compare NO production by fibroblasts derived from HSc tissue and normal skin (Chapter Four).
4. To compare TGF-β mRNA expression and protein production by HSc tissue and cells with those by normal skin and fibroblasts (Chapter Five).
5. To compare TGF-β mRNA and protein production by HSc and normal fibroblasts after IFN treatment with those untreated controls Chapter Six).
6. To examine TGF-β mRNA in HSc tissues and protein in the serum of HSc patients after IFN-α2b treatment (Chapter Six).

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CHAPTER THREE

HUMAN DERMAL FIBROBLASTS PRODUCE NITRIC OXIDE AND EXPRESS BOTH CONSTITUTIVE AND INDUCIBLE NITRIC OXIDE SYNTHASE ISOFORMS*

3.1 INTRODUCTION

Nitric oxide (NO), originally identified as a blood vessel relaxing factor derived from endothelial cells (Palmer et al, 1987; 1988), has been shown to possess a variety of physiological and pathophysiological activities including vasodilation (Humphreys et al, 1991; Benyo et al, 1991; Kovach et al, 1992), antimicrobial (Hibbs et al, 1988), antitumor (Stuehr and Nathan, 1989), immunoregulation (Schneemann et al, 1993; Green and Nacy, 1993) and functioning as a neuro-transmitter (Snyder and Bredt, 1992). The biosynthesis of NO is mediated by NO synthases (NOS), β-nicotinamide adenine dinucleotide phosphate, reduced form-dependent enzymes that catalyze the oxidation of one of the guanidino nitrogen atoms of L-arginine with molecular oxygen to form NO and L-citrulline (Kwon et al, 1990; Leone et al, 1991). Three isoforms of NOS have been distinguished by cloning of their genomic and complementary DNA in several mammalian species including humans (Bredt et al, 1991; Geller et al, 1993; Marsden et al, 1992; Nakane et al, 1993; Xie et al, 1992). Activities of two of these isoforms are dependent on elevated intracellular Ca^{2+} and exogenous calmodulin and are constitutively expressed (eNOS). One (endothelial NOS, eNOS) appears to be restricted to endothelial cells while another is expressed in neurons (nNOS) of the central and peripheral nerve system and in skeletal muscles (Nakane et al, 1993). The third isoform, inducible NOS (iNOS), is independent of elevated intracellular Ca^{2+} and possesses its own calmodulin as a tightly bound subunit (Cho et al, 1992). This isoform is not expressed under normal conditions but can be induced in many types of cell by inflammatory cytokines and bacterial LPS.

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In humans, as in other mammals (Nathan, 1992), many types of cell have been shown to synthesize NO and express NOS (Nussler and Billiar, 1993; Moncada et al, 1991; Taylor-Robinson et al, 1994). However, the amount of NO produced by human cells is much lower than that by rodent cells and there is some controversy over whether or not human monocytes/macrophages produce NO (Denis, 1994). Under normal conditions, fibroblasts are responsible for tissue integrity and extracellular matrix metabolism by producing structural (e.g. collagens) and functional (e.g. collagenase) proteins. In damaged tissue, fibroblasts actively migrate towards the wound site, proliferate, and produce more extracellular matrix proteins to facilitate wound healing. Activated fibroblasts in injured tissues also produce cytokines such interleukin-1 (di Giovine and Duff , 1990), interleukin-6 (Willis et al, 1994) and IFN-β and -α (Austyn and Wood, 1993), which further augment the inflammatory response. Rat and mouse fibroblasts have been shown to produce NO upon stimulation with cytokines and LPS (Jorens et al, 1992; Werner-Felmayer et al, 1990), suggesting that NO produced by fibroblasts may play a role in regulating the process of inflammation and wound healing, possibly through its vasodilator and antiproliferative effects (Jorens et al, 1992; Willis et al, 1994).

Although production of NO by rodent fibroblasts has been well-studied, it is not known whether human fibroblasts express NOS or produce NO. In this report, we provide evidence that human fibroblasts, in addition to the production of extracellular matrix proteins, express NOS and synthesize NO, suggesting that they may participate in regulating the inflammatory and healing process of wounds by synthesizing and releasing NO, which may play a role in wound healing and scar tissue remodeling.

3.2 MATERIALS AND METHODS

3.2.1 Cell Culture

Human dermal fibroblasts were isolated from punch biopsies of normal human skin by the standard explant procedure described previously (Tredget et al, 1993). The cells were cultured in Dulbecco's modified Eagle medium (DMEM; arginine concentration 400 μM; GIBCO BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 10 ml/L of Antibiotic-Antimycotic (100 X; GIBCO BRL), 20 mM of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 3.7 g/L of $NaHCO_3$ (Sigma, St. Louis, MO). The cells were incubated at 37°C in an atmosphere of 5% CO_2 in air with 95% relative humidity. After passage 4 following the explant, the resulting cell population consisted of more than 98% fibroblasts as assessed by morphological criteria, typical aging profile, growth characteristics and uniformly positive Thy-1 immunostaining (monoclonal antibody against human Thy-1; provided by Dr. J. Fabre, University of London, London, England; McKenzie and Fabre, 1981). Six cell strains from 6 different individual punch biopsies (uninjured skin from post-burn hypertrophic scar patients; Ghahary et al, 1992) were used in this study. All experiments were carried out with cell cultures at passages 5 to 9.

3.2.2 Spectrophotometric Estimation of Nitric Oxide Produced by Human Dermal Fibroblasts

To measure nitrite production by human dermal fibroblasts, confluent cells in 75 cm^2 tissue culture flasks were trypsinized, washed and resuspended in supplemented DMEM (10% FCS, Hyclone Laboratories, Logan, UT). The cells were counted using a Coulter Counter (Coulter Electronics, Inc. Hialeah, FL) and seeded into 24-well tissue culture plates (Corning Incorporated, Corning, NY) at a density of 3 to 5 X 10^5 cells/well. After incubation for 12 hr, the cells in quadruplicate wells of 24 well plates were treated with different concentrations of human recombinant IFN-γ (hrIFN-γ; Hoffmann-La Roche Inc. Nutley, NJ), LPS (from *Pseudomonas aeruginosa*, serotype 10; Sigma) or a combination of both. After incubation at 37°C for different periods of time, nitrite was determined in the cell-free supernatant by a spectrophotometric method based on the Griess reaction (Green et al, 1990). The absorbency at 550 nm was measured using a microplate reader (Model EAR 400AT; SLT-Labinstruments, Grödig, Austria). The nitrite concentration was calculated from a $NaNO_2$ (Sigma) standard curve, corrected for the cell number, and expressed as nmol/10^6 cells/96 hrs. Background levels of nitrite were determined in cell-free DMEM with or without the additives and were subtracted from the total amount of nitrite formed. The detection limit for nitrite was 1 nmol/ml. For positive controls, J774A.1 cells, a mouse monocyte/macrophage cell line from American Type Culture Collection, were stimulated with 50 U/ml murine recombinant tumor necrosis factor-α (mrTNF-α) plus 10 μg/ml LPS and production of NO was determined in parallel (Fujihara et al, 1994).

3.2.3 Effect of L-NMMA, L- or D-arginine on Nitric Oxide Production of Human Dermal Fibroblasts

To determine the effect of L-NMMA, a competitive inhibitor of NOS, on the nitrite production of human dermal fibroblasts, the cells in quadruplicate wells of 24-well plates were treated with 200 U/ml hrIFN-γ, 40 μg/ml LPS and different concentrations of L-NMMA (acetate salt, Calbiochem, La Jolla, CA) for 96 hrs. The cell-free supernatants were collected and analyzed for nitrite. To test the effect of L- or D-arginine on restoring nitrite production by human dermal fibroblasts inhibited by L-NMMA, the cells in quadruplicate wells of 24-well plates were treated with hrIFN-γ, LPS, 250 μM of L-NMMA, and different concentrations of L- or D-arginine for 96 hrs. The cell-free culture media with or without the additives were also assessed for background NO formation, which was subtracted from the total.

3.2.4 Detection of Ca^{2+}-Dependent and -Independent NOS Activities

The Ca^{2+}-dependent and -independent NOS activities in the cytosol of human dermal fibroblasts were determined by measuring the conversion of [^{14}C]L-arginine to [^{14}C]L-citrulline as described previously (Knowles et al, 1990). The protein content of the cell cytosol was determined by the Bradford dye-binding procedure (Bio-Rad) using bovine serum albumin as a standard. The Ca^{2+}-dependent NOS activity was defined as the EGTA-inhibitable conversion of L-[U-^{14}C]arginine to L-[U-^{14}C]citrulline by the cell cytosol. The Ca^{2+}-independent

NOS activity was determined from the difference between the L-NMMA-inhibitable and L-NMMA-EGTA-inhibitable activities measured using the conversion of the radioactive L-arginine to L-citrulline. For the Ca^{2+}-dependent NOS positive control, rat brains were washed with PBS, homogenized and assessed for NOS activities. For the Ca^{2+}-independent NOS positive control, J774A.1 cells were stimulated with 50 U/ml mrTNF-α and 10 μg/ml LPS for 18 hrs and the cytosol was analyzed for NOS activity in parallel with the cytosol from human dermal fibroblasts.

3.2.5 Detection of mRNAs for eNOS and iNOS in Human Dermal Fibroblasts Using Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from stimulated and control cells using the guanidinium isothiocyanate (GITC)/CsCl procedure of Chirgwin et al. (1979). Five microgram of the isolated RNA was run on a 1% agarose gel containing 1μg/ml ethidium bromide to confirm that there was no degradation of the RNA. First-strand cDNA (RT-cDNA) was made from total RNA by using Superscript RNase H^- reverse transcriptase (Superscript RT II; GIBCO BRL) and Oligo $(dT)_{12-18}$ Primer (GIBCO BRL) as primer, using a conventional procedure (Reiling et al, 1994).

The PCR was carried out in an automatic DNA thermal cycler (Model TR 96, Tyler Research Instrument Corporation, Edmonton, Canada). The following gene-specific primers were used to amplify the desired cDNA: eNOS sense (5'-GTG ATG GCG AAG CGA GTG AAG-3') and eNOS antisense (5'-CCG AGC CCG AAC ACA CAG AAC-3'), which correspond to nucleotides 39-59 and 440-460 of the human umbilical vein endothelial cell eNOS cDNA, respectively; iNOS sense (5'-TCC GAG GCA AAC AGC ACA TTC A-3') and iNOS antisense (5'-

GGG TTG GGG GTG TGG TGA TGT-3'), which correspond to nucleotides 2263-2284 and 2704-2724 of human hepatocyte iNOS cDNA, respectively. The primers were synthesized in the Nucleic Acid Research Center, University of Alberta. The reaction mixture contained reaction buffer with 2.5 mM $MgCl_2$, 100 μM of each of the dNTPs, 0.5 μM sense and antisense primers, 2.5 units Taq DNA polymerase (Perkin-Elmer) and different concentrations of RT-cDNA in a final volume of 50 μl. The PCR conditions were 30 seconds of denaturing at 95°C, 60 seconds of annealing at 59°C, and 90 seconds of extension at 73°C for 35 cycles. The amplified products were analyzed on a 1% agarose (Bio-Rad) gel containing 1 μg/ml ethidium bromide. The sizes of the PCR products were determined by calculating the base pairs between the sense and anti-sense primers aligned with the corresponding human endothelial eNOS and hepatocyte iNOS cDNA sequences and indicated by the 100 base pair (bp) DNA molecular weight marker (GIBCO BRL). For positive controls, the plasmid cDNAs specific for human eNOS and hepatocyte iNOS were used as templates in PCR. For negative controls, total cellular RNA from human dermal fibroblasts without reverse transcription was used as templates in PCR.

3.2.6 Detection of eNOS and iNOS Proteins in Human Dermal Fibroblasts Using Immunocytochemistry

A mouse peroxidase anti-peroxidase (Sigma) immunostaining procedure was used to detect eNOS and iNOS in human dermal fibroblasts. For eNOS immunostaining, treated (IFN-γ and LPS for 24 hrs) or untreated fibroblasts (2.5×10^4) on glass microscope slides were washed with PBS and fixed in 4% paraformaldehyde. The endogenous peroxidase was inactivated by incubating the cells with 50%

methanol in Milli-Q H_2O containing 0.16 μM H_2O_2 (Fisher). After washing and permeabilizing the cells with 0.05% polyoxyethylene sorbitan (Tween 20; Fisher) in PBS, nonspecific binding was blocked with 10% normal goat serum. The cells were then incubated with monoclonal antibody (mAb) against human endothelial eNOS (10 μg/ml; Transduction Laboratories, Lexington, KY) for 18 hrs at 4°C. The slides were washed with PBS and incubated with a 1:100 dilution of bridging antibody (goat-anti-mouse IgG; Sigma). After washing with PBS 5 times, the slides were treated with peroxidase anti-peroxidase complex (Sigma), incubated with substrate solution (5 μg/ml of 3,3'-diaminobenzidine in 100 mM Tris buffer, pH 7.2, containing 1 μl/ml of 30% H_2O_2), and lightly counterstained with Harris' modified hematoxylin (Fisher). In negative controls, the primary antibody was replaced with nonimmune normal mouse IgG. For iNOS immunostaining, treated and untreated fibroblasts on slides were stained using the same procedure as for eNOS except for that a mAb against mouse macrophage iNOS (10 μg/ml in PBS containing 0.2% BSA; Transduction Laboratories) was used as the primary antibody. To examine NOS protein expression by fibroblast-like cells in dermal tissue, 5 μm paraffin sections prepared from normal skin biopsies were stained with eNOS and iNOS antibodies using the above procedure. After immunostaining, the slides were examined and microphotographs were taken using a Nikon OPTIPHOT-2 microscope equipped with a FX-35DX camera (Nikon Optical, Tokyo, Japan).

3.3 RESULTS

3.3.1 Human Dermal Fibroblasts Spontaneously Produce Nitric Oxide, Which Is Increased by IFN-γ and LPS

Human dermal fibroblasts were stimulated with hrIFN-γ and LPS alone and in combination. Without any stimulation, the fibroblasts produced a small amount of NO. Neither IFN-γ nor LPS alone significantly stimulated the production of NO, however, addition of IFN-γ and LPS together did (**Figure III-1A**). Human dermal fibroblasts showed dose-dependent responses to hrIFN-γ and LPS, as determined by the spectrophotometric assay (data not shown). The combination of 200 U/ml of hrIFN-γ and 40 μg/ml of LPS induced maximum NO production by the cells and these concentrations were, therefore, used in all subsequent experiments.

Figure III-1B shows that NO production by IFN-γ/LPS treated cells is time dependent. As shown in this figure the NO production is markedly higher in treated cells at any given time. The dynamic change of nitrite in the media with time reflects a balance between NO production of the cells and degradation of nitrite. The continual increases during the first 4 days are due to higher rate of NO synthesis resulting in accumulation of nitrite in conditioned media. The decreases found afterwards are due to reduced NO synthesis of the cells after long period of incubation in the same media.

To determine NO production by different strains of human dermal fibroblasts, cells derived from 6 individuals were cultured in 24-well plates and stimulated with hrIFN-γ and LPS. All strains of human dermal fibroblasts tested produced NO and 5 out of 6 strains produced significantly higher levels of NO with stimulation (data not shown). As a positive control, the production of NO by the mouse monocyte/macrophage cell line J774A.1 cells was examined using the Griess reaction. Without stimulation, J774A.1 cells produced NO at a level similar to human dermal fibroblasts. However, when the cells were treated with mrTNF-α and LPS, they produced much higher levels of NO than human dermal fibroblasts (**Table III-1**).

3.3.2 Nitric Oxide Production by Human Dermal Fibroblasts Is Blocked by the Competitive NOS Inhibitor L-NMMA

L-NMMA inhibited NO production by the cells in a dose-dependent manner (**Figure III-2A**). This inhibition was not due to cytotoxicity since L-NMMA had no effect on the viability of the cells as determined by the trypan blue dye exclusion test (data not shown) and its effect could be reversed by L-arginine but not by D-arginine (**Figure III-2B**). The control groups in Figure 2B were stimulated with IFN-γ and LPS without L-NMMA.

3.3.3 Human Dermal Fibroblasts Express Both Ca^{2+}-Dependent and Ca^{2+}-Independent NOS Activities

Table III-2 shows the Ca^{2+}-dependent and Ca^{2+}-independent NOS activities from cytosolic extraction of rat brain, mrTNF-α/LPS-treated and untreated J774A.1 cells and hrIFN-γ/LPS-stimulated and unstimulated human dermal fibroblasts as measured by the citrulline assay. Rat brain and activated J774A.1 cells were used as positive controls for determining Ca^{2+}-dependent and -independent NOS activities because they are well-known to express eNOS and iNOS, respectively (Bredt et al, 1991; Knowles et al, 1990). Ca^{2+}-dependent NOS activity was detected in the cytosolic extractions from both stimulated and unstimulated human dermal fibroblasts, but the Ca^{2+}-independent NOS activity was only detected in stimulated cells (**Table III-2**).

3.3.4 Human Dermal Fibroblasts Express mRNAs for Both eNOS and iNOS

Since both Ca^{2+}-dependent and -independent NOS activities are observed in human dermal fibroblasts, we examined the expression of mRNAs for eNOS (Marsden et al, 1993) and iNOS (Geller et al, 1993) in the cells using RT-PCR. **Figure III-3** shows the ethidium bromide-stained PCR products in an agarose gel. Lanes 1 and 7 show amplification of eNOS and iNOS using corresponding plasmid cDNAs as templates in PCR for positive controls. The 422 bp product for endothelial eNOS and the 462 bp product for iNOS are the correct sizes for these products as indicated by the DNA ladder (lane M). When the total cellular RNA without reverse transcription was used as templates in PCR, the bands for eNOS and iNOS were not apparent (lane 2 and 8). The intensity of the bands increased with increasing amounts of RT-cDNA (Lane 3-6 for eNOS and 9-12 for iNOS). These results show that human dermal fibroblasts express mRNAs for both eNOS and iNOS.

3.3.5 Human Dermal Fibroblasts Express eNOS and iNOS Proteins *in vitro*

Under low magnification, almost all of the fibroblasts appeared to be stained with the eNOS antibody, although some showed relatively intense staining while the others showed rather weak staining (**Figure III-4A**). Under high magnification, the cytoplasm around the nuclei of the cells had stronger staining in the intensely stained fibroblasts, but the staining in other cells was scattered throughout in the cytoplasm (**Figure III-4B**). As shown in **Figure III-4C**, unstimulated control cells and stimulated control cells (data not shown) did not show any staining when the primary antibody was replaced with non-immune mouse IgG. **Figure III-4D** shows that only some of the IFN-γ and LPS treated cells demonstrated intense staining, while most showed faint staining. The strongly stained area in the stimulated cells was in the cytoplasm close to the nuclei (**Figure III-4E**). When the primary antibody was replaced with non-immune mouse IgG, the stimulated control cells (**Figure III-4F**) and unstimulated control cells (data not shown) did not show any staining.

3.3.6 Fibroblast-like Cells in Normal Human Dermis Express eNOS

After eNOS immunostaining, some of the fibroblast-like cells and blood vessels in the dermis are positive under low magnification (**Figure III-5A**). Under high magnification, some of the fibroblast-like cells showed stronger staining (**Figure III-5B**; arrows). The cytoplasm close to the nuclei showed stronger staining in the intensely stained cells. Endothelial cells of blood vessel are also intensely stained with eNOS antibody (data not shown). **Figure III-5C** and III-**5D** show that all cells in the dermis are negative after iNOS immunostaining. When the primary antibodies were replaced by non-immune mouse IgG, none of the cells in the dermis showed any staining (**Figure III-5E** and **-5F**).

3.4 DISCUSSION

The main interest and rapid progress in NO research is attributed to its diversity of physiological and pathophysiological functions and synthesis by a variety of cell types (Nathan, 1992). Besides its vasodilatory effect and intercellular messenger functions, NO is well-known to play roles in the inflammatory response and immunoregulation (Moncada et al, 1991). In this study, we demonstrated that fibroblasts derived from normal human skin spontaneously synthesized NO in culture and that this production was enhanced by stimulation with hrIFN-γ and LPS. Synthesis of NO by dermal fibroblasts was characteristic of both constitutive and inducible responses as described in cultured rat aortic endothelial cells (Suschek et al, 1993), since the cells produced relatively low amounts of NO without stimulation and the production was significantly increased when stimulated with hrIFN-γ and LPS. The inducible NO synthesis has been reported in rat lung fibroblasts (Jorens et al, 1992) and murine dermal fibroblasts (Werner-Felmayer et al, 1990). The amount of NO produced by human dermal fibroblasts appears comparable to that produced by rodent fibroblasts (Jorens et al, 1992; Werner-Felmayer et al, 1990). The physiological significance of the inducible NO production in fibroblasts is unknown, but it has been speculated that it could play a important role in regulating the inflammatory response to tissue injury (Jorens et al, 1992).

The constitutive synthesis of NO by human fibroblasts has not, to our knowledge, been previously reported. Here, we demonstrate that human fibroblasts derived from normal skin are capable of constitutively synthesizing NO in culture. The constitutive and inducible production of NO by the fibroblasts was further confirmed by subsequent experiments showing that the cell cytosol from the fibroblasts have both Ca^{2+}-dependent and -independent NOS activities, and that the fibroblasts express both eNOS and iNOS mRNAs and proteins. The physiological significance of constitutive production by human fibroblasts is not clear. However, this low level of NO constitutively released by the fibroblasts could possibly play a role in regulating the blood flow in healing wounds, directly influencing the wound healing process.

To confirm that human fibroblasts, like other types of cells, use the L-arginine/NO pathway, we tested the effects of the well-characterized NOS inhibitor L-NMMA (Rees et al, 1990). Because L-NMMA inhibits the NOS activity by competing with the normal substrate L-arginine, addition of excess L-arginine should restore the production of NO. The data showed that the NO production by the fibroblasts is indeed inhibited by L-NMMA and addition of excess L-arginine but not D-arginine restored it, demonstrating that the cells specifically use the L-arginine/NO pathway. These results are in agreement with the inhibition of NO synthesis by L-NMMA in rat lung fibroblasts (Jorens et al, 1992) and murine dermal fibroblasts (Werner-Felmayer et al, 1990). This is not surprising since all types of cells studied in mammals use this pathway in NO synthesis.

The results of the radioactive citrulline assay showed that the cell cytosol possessed both Ca^{2+}-dependent and -independent NOS activities suggesting that human fibroblasts expressed both eNOS and iNOS. As expected the Ca^{2+}-dependent NOS activity was not significantly influenced by stimulation with IFN-γ and LPS. The Ca^{2+}-dependent NOS activity in the fibroblasts was lower than that in pig endocardial cells (Schulz et al, 1991) or human umbilical vein endothelial cells (Rosenkranz-Weiss et al, 1994) but was in the same range (0.5 to 5.0 pmol/mg protein/min). It was of interest that the J774A.1 cells also showed Ca^{2+}-dependent NOS activity in the same range. Ca^{2+}-dependent NOS activity has not been previously reported in these macrophages, however, this finding supported the previous evidence that human monocytes/macrophages expressed eNOS mRNA (Reiling et al, 1994). The level of Ca^{2+}-independent NOS activity in the cell cytosol was similar to that found in rat myocardial cells (Schulz et al, 1992), human B lymphocytes (Mannick et al, 1994), and human keratinocytes (Heck et al, 1992), but was much lower than that in rat (Griscavage et al, 1993) or murine (Vodovotz et al, 1993) macrophages.

To confirm that the Ca^{2+}-dependent and -independent NOS activities in human dermal fibroblasts are due to eNOS and iNOS, we examined the eNOS and iNOS mRNAs in the cells. Because of the low levels of NO production and low levels of NOS activities in human dermal fibroblasts, we used RT-PCR to detect the mRNAs. The sequences of the oligonucleotide primers used in this study are the same as those used in the RT-PCR procedure for detection of eNOS and iNOS mRNAs in human monocytes/macrophages (Reiling et al, 1994). The 422 and 462 bp RT-PCR products derived from human monocytes/macrophages showed more than 99% and 99% identity to human eNOS and human hepatocyte iNOS cDNA, respectively (Reiling et al, 1994). Thus, we believe that the 422 and 462 bp RT-PCR products from human dermal fibroblasts are identical to human eNOS and human hepatocyte iNOS. Our data indicate that human dermal fibroblasts express the mRNAs for both eNOS and iNOS.

The final corroboration of eNOS and iNOS in human dermal fibroblasts comes from immunocytochemistry. The results demonstrate that both stimulated and unstimulated cells express eNOS protein while the stimulated cells express more iNOS protein than unstimulated cells. Some unstimulated cells also showed faint staining with anti-iNOS antibody. This may due to low levels of activation of the cells by the culture conditions (24 hrs on slides, not in culture flasks) and/or a weak cross-reaction between the mouse macrophage iNOS antibody and human eNOS. Some of the fibroblast-like cells in normal dermis are stained with eNOS antibody suggesting that these cells may express eNOS and produce NO in vivo. However, all the cells in normal dermis are negative when stained with iNOS antibody. This may be due to lack of proper induction of the enzyme in normal skin.

The significance of NO production by human dermal fibroblasts is not clear. It can be speculated that, in the wound, the constitutive low levels of NO produced by fibroblasts, along with the NO produced by vascular endothelial cells, maintain a higher rate of blood flow in the wound. The increased blood flow would facilitate both nutrition and cell infiltration, which benefit wound healing. Furthermore, this low level of NO produced by fibroblasts could neutralize the oxygen free radicals released by infiltrated leukocytes, thus, protecting cells from damage by oxygen and hydroxyl radicals (Harbrecht et al, 1992). On the other hand, cytokines released by infiltrated leukocytes and LPS associated with bacterial invasion could stimulate neutrophils, macrophages and the wound fibroblasts to produce large amounts of NO, which in turn might inhibit the cytokine and NO production by the infiltrated leukocytes (Griscavage et al, 1993), and reduce cell proliferation (Firnhaber et al, 1993). This could provide a negative regulation of the inflammatory responses of the wound, since severe inflammation and high levels of NO can cause massive tissue damage which is detrimental to wound healing. Interestingly, transforming growth factor-β (TGF-β), known to be a potent fibrogenic cytokine that enhances wound healing, has been reported to inhibit NO production of macrophages (Vodovotz et al, 1993), suggesting that high levels of NO may inhibit the wound healing process.

In conclusion, human dermal fibroblasts spontaneously produce NO in culture and this production is increased upon stimulation with IFNγ and LPS. The constitutive and inducible NO synthesis by the cells is mediated by Ca^{2+}-dependent and -independent NOS activities corresponding to eNOS and iNOS. From these findings, we speculate that human dermal fibroblasts may be involved in the inflammatory response to skin injury and actively participate in wound healing and scar tissue remodeling.

Table III-1. **Human Dermal Fibroblasts and J774A.1 Cells Produce Nitrite as Detected with Griess Reaction.**

		NITRITE[a] (nmol/10^6 cells/96 hrs ± SEM)
FIBROBLASTS	Stimulated	23.43 ± 2.78[b]
	Unstimulated	12.46 ± 1.96
J774A.1 CELLS	Stimulated	297.02 ± 27.90[c]
	Unstimulated	9.64 ± 2.74

[a] Measured using the spectrophotometric procedure based on Griess reaction as described in "Materials and Methods".

[b] Significantly higher than unstimulated controls ($p = 0.005$; $n = 8$; t-test).

[c] Significantly higher than unstimulated controls ($p < 0.001$; $n = 8$; t-test).

Table III-2. **NOS Activities Are Present in Human Dermal Fibroblasts, J774A.1 Cells and Rat Brain.**

		NOS ACTIVITIES[a]	
		Ca^{2+}-Dependent	Ca^{2+}-Independent
FIBROBLASTS	Stimulated (n = 4)	2.60 ± 0.54	1.59 ± 0.14
	Unstimulated (n = 4)	1.36 ± 0.57	0.06 ± 0.44
J774A.1 CELLS	Stimulated (n = 4)	9.67 ± 2.52	19.05 ± 3.96
	Unstimulated (n = 4)	2.27 ± 0.83	N. D.[b]
RAT BRAIN	(n = 4)	2.86 ± 0.29	N. D.

[a] Determined using the radioactive citrulline assay as described in "Materials and Methods" and expressed as pmol of L-arginine converted by NOS per mg of protein in the cell cytosol per min (pmol/mg/min ± SEM).

[b] N. D.: not detectable.

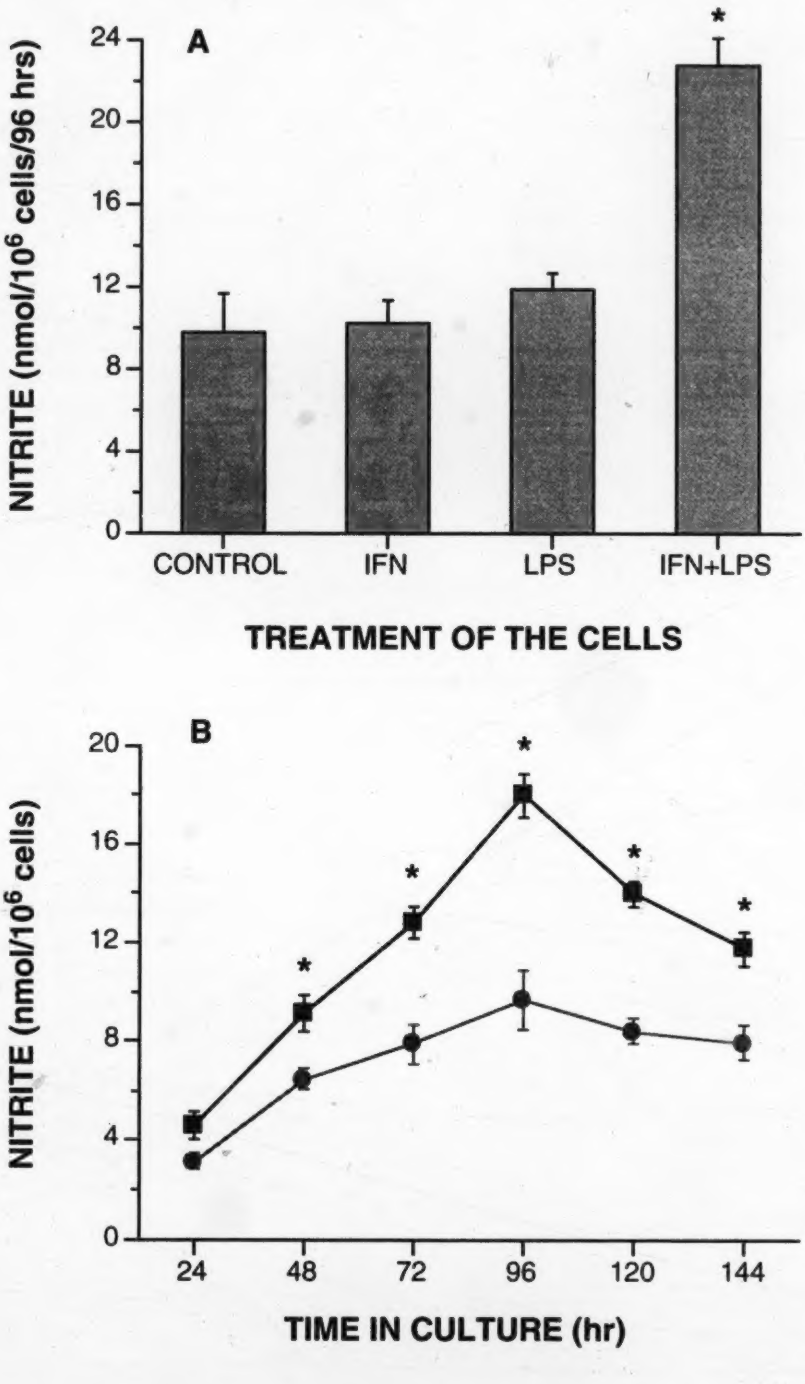

Figure III-1. See legend on next page.

Figure III-1. **Human dermal fibroblasts produce NO as detected by Griess reaction:** Confluent cells in 24-well plates were treated with hrIFN-γ and LPS either individually or in combination. Nitrite in the cell-free supernatants was measured using the procedures described in "Materials and Methods". (*A*), *Synergistic effect of IFN-γ and LPS on NO production by human fibroblasts*. The cells were treated with PBS (control), hrIFN-γ alone, LPS alone or hrIFN-γ plus LPS for 96 hrs. (*B*), *Time course of NO production by human dermal fibroblasts*. The cells were treated with hrIFN-γ plus LPS (—■—) or incubated in media containing PBS (—●—). Data for A and B represent mean ± SEM obtained from 3 separate experiments. Stars (*) indicate the significant difference between IFN-γ plus LPS-treated cells and the other groups.

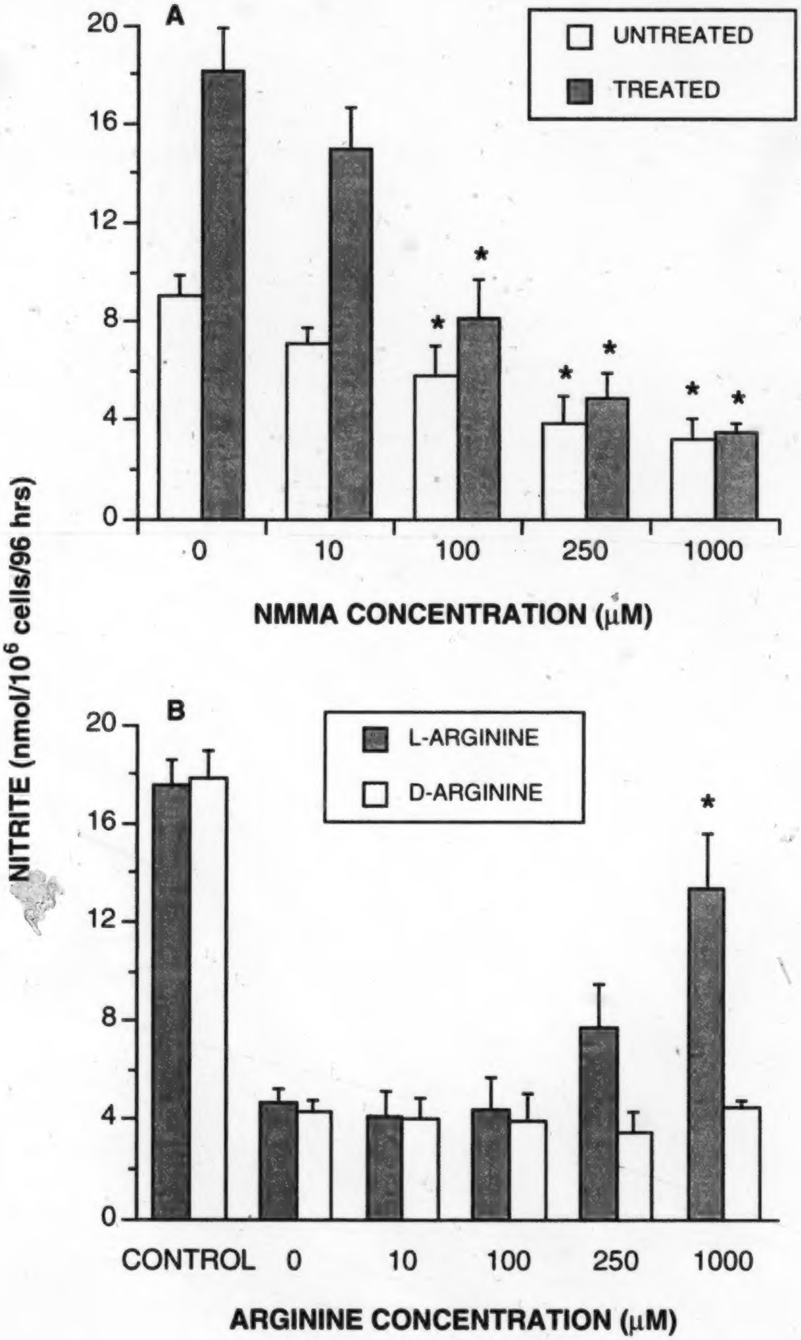

Figure III-2. See legend on next page.

Figure III-2. **NO production by fibroblasts is inhibited by L-NMMA, which is reversed by L- but not D-arginine:** (*A*), *Dose-dependent inhibition of NO production of human fibroblasts by L-NMMA*. The IFN-γ/LPS stimulated and unstimulated cells were treated with different concentrations of L-NMMA for 96 hrs. Nitrite in the supernatants were measured with the Griess reaction. (*B*), *Dose-dependent restoration of NO production by human fibroblasts by L-arginine*. The IFN-γ/LPS stimulated cells were treated with 250 mM of L-NMMA and different concentrations of L- (open column) or D-arginine (closed column) for 96 hrs. Data for A and B represent mean ± SEM obtained from 3 separate experiments. Stars (*) indicate a significant difference between L-NMMA treated and control cells for stimulated (closed column) and unstimulated (open column) cells or between L- (open column), D-arginine (closed column) treated and control cells.

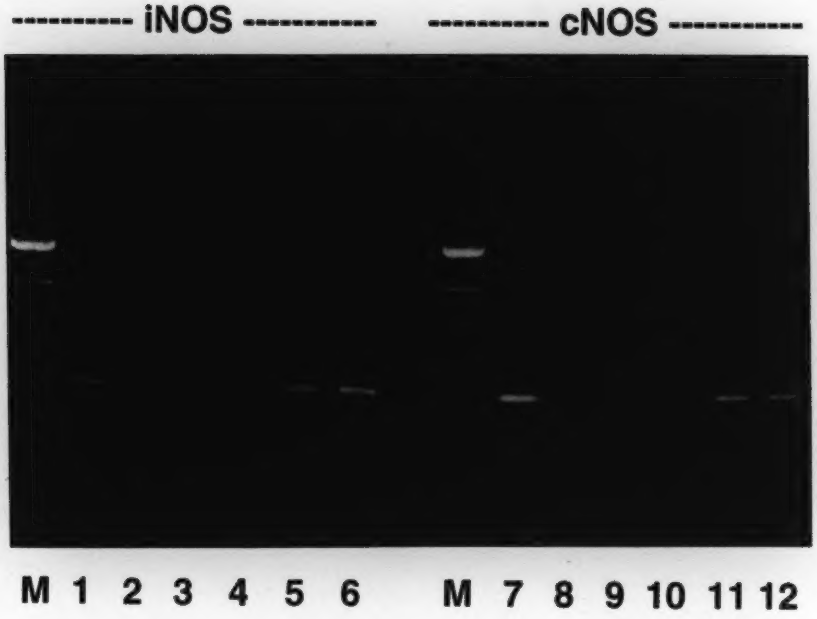

Figure III-3. **Human dermal fibroblasts express cNOS and iNOS mRNAs:** RT-PCR was used to detect mRNAs for iNOS (lane 2-6) and cNOS (lane 8-12). Photograph represents the iNOS and cNOS PCR products stained by ethidium bromide. Lanes M show the 100 bp DNA ladder. Lanes 1 and 7 show positive controls using plasmid cDNA for iNOS and cNOS, respectively. Lanes 2 and 8 show the negative controls using RNA without reverse transcription for iNOS and cNOS. Lanes 3 to 6 are iNOS products obtained from various amounts of RT-cDNA (1, 5, 25 and 125 ng/reaction) used in PCR. Lanes 9 to 12 are cNOS products obtained from various amounts of RT-cDNA (1, 5, 25 and 125 ng/reaction) used in PCR.

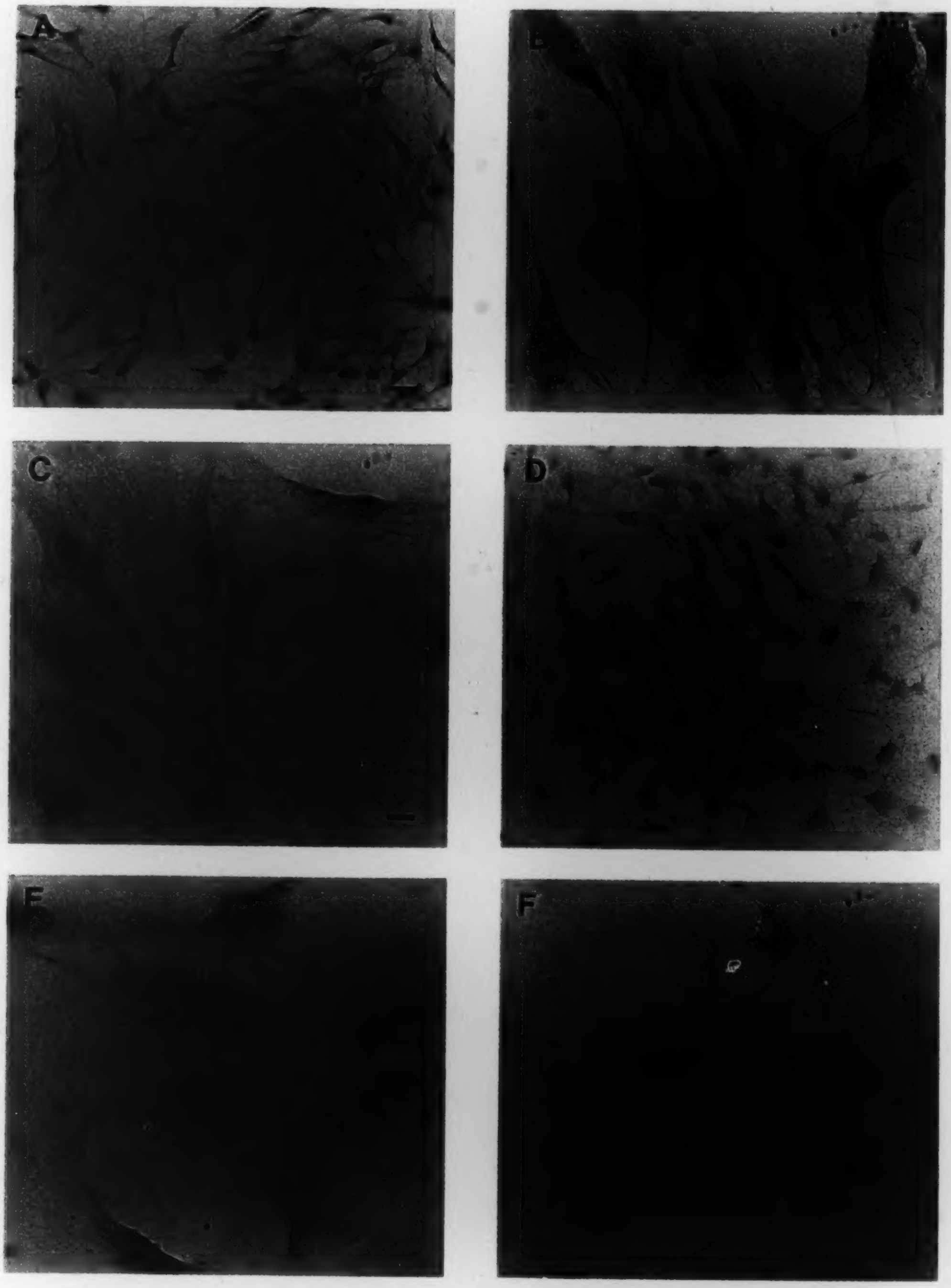

Figure III-4. See legend on next page.

Figure III-4. **Human dermal fibroblasts express eNOS and iNOS proteins as determined by immunocytochemistry:** Stimulated and unstimulated cells were stained with a mouse peroxidase anti-peroxidase procedure to visualize eNOS and iNOS proteins. Panels A to C show the eNOS immunostaining of the unstimulated cells and panels D to F show the iNOS immunostaining of stimulated cells. (*A*), *Low-power view of eNOS immunostaining*. The bar indicates 8 μm for both A and D. (*B*), *High-power view of eNOS immunostaining*. Arrows indicate granules in the cytoplasm of strongly stained cells. (*C*), *Control for eNOS*. In the control, the primary antibody was replaced by nonimmune mouse IgG. The bar indicates 2 μm for B, C, E, and F. (*D*), *Low-power view of iNOS immunostaining*. The cells on microscope slides were stimulated with IFN-γ plus LPS, fixed and stained with iNOS antibody. (*E*), *High-power view of iNOS immunostaining*. Arrow indicates granules in the cytoplasm of the intensively stained cells (*F*), *Control for iNOS immunostaining*. The cells on slides were treated with IFN-γ plus LPS and subjected to the immunostaining but the primary antibody was replaced by nonimmune mouse IgG.

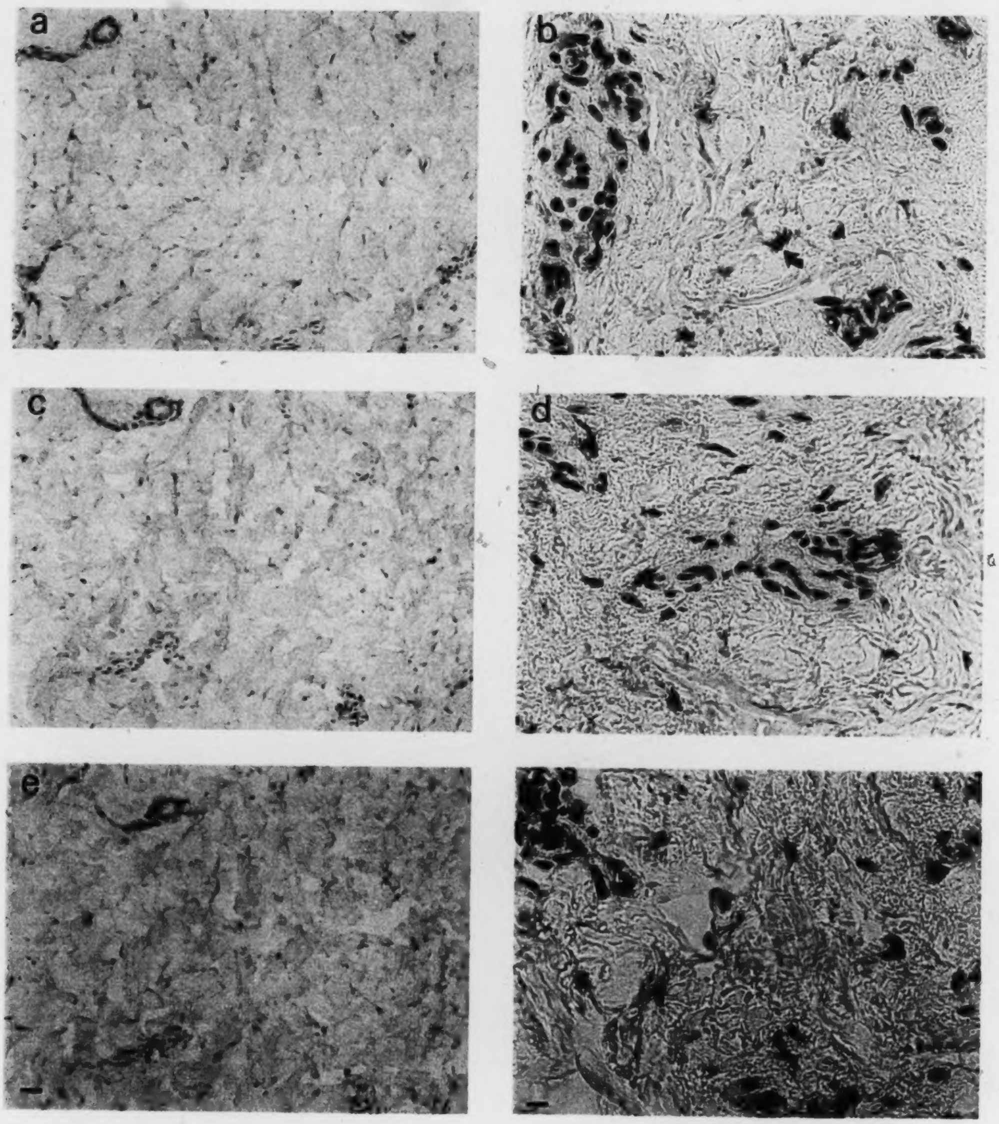

Figure III-5. See legend on next page.

Figure III-5. **Fibroblast-like cells in human dermis express eNOS proteins as determined by immunocytochemistry:** Paraffin-embedded sections from human skin punch biopsies were stained with a mouse peroxidase anti-peroxidase procedure to visualize eNOS and iNOS proteins. Panels (a), (b) and (c), (d) show the eNOS and iNOS immunostaining, respectively. Panels (e) and (f) show the negative controls, in which the primary antibodies were replaced by non-immune mouse IgG. (*a*), *Low-power view of eNOS immunostaining*. Some cells in the dermis and the blood vessels showed positive staining. (*b*), *High-power view of eNOS immunostaining*. Some of the fibroblast-like cells in the dermis are stained with eNOS antibody. Arrows indicate granules in the cytoplasm of strongly stained fibroblast-like cells. (*c*), *Low-power view of iNOS immunostaining*. No positive stained cells in the dermis. The bar indicates 10 μm for panels (a), (c) and (e). (*d*), *High-power view of iNOS immunostaining*. All the fibroblast-like cells and other type of cells are negative. (*e*), *Low-power view of negative control*. (*f*), *High-power view of negative control*. All the cells in the dermis are negative. The bar indicates 2.5 μm for panels (b), (d) and (f).

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CHAPTER FOUR

NITRIC OXIDE SYNTHASE EXPRESSION AND NITRIC OXIDE PRODUCTION ARE REDUCED IN HYPERTROPHIC SCAR TISSUE AND FIBROBLASTS*

4.1 INTRODUCTION

Nitric oxide, a bioactive product of NO synthases (NOS) in the arginine-citrulline metabolic pathway, has been shown to possess several physiological and pathophysiological activities. These include smooth muscle relaxation or vasodilatation (Benyo et al, 1991; Humphreys et al, 1991), microbicidal activity (Green et al, 1990; Hibbs et al, 1988), tumoricidal activity (Lorsbach et al, 1993; Stuehr and Nathan, 1989), immune regulation (Schneemann et al, 1993; Taylor-Robinson et al, 1994), anti-proliferation (Nunokawa and Tanaka, 1992; Stein et al, 1995), and neurotransmission (Przewlocka et al, 1994; Snyder and Bredt, 1992). The biosynthesis of NO is mediated by NOS, β-nicotinamide adenine dinucleotide phosphate (reduced form)-dependent enzymes with 3 isoforms (Geller et al, 1993; Marsden et al, 1992; Nakane et al, 1993). Two of these isoforms (endothelial NOS or eNOS and neuronal NOS or nNOS) are constitutively expressed, and their activities are dependent on elevated intracellular Ca^{2+} and extrinsic calmodulin (Bredt and Snyder, 1990; Zhang and Vogel, 1994). The third isoform is not expressed under normal conditions but can be induced in many types of cells by inflammatory cytokines and bacterial lipopolysaccharide (LPS). Its activity is independent of elevated intracellular Ca^{2+}, and it possesses calmodulin as a tightly bound subunit (Cho et al, 1992).

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Hypertrophic scarring (HSc), a fibroproliferative disorder of the dermis, occurs after wounding, particularly after severe thermal injury (Boykin and Molnar, 1992; Scott et al, 1994). HSc is characterized by raised, red, nodular and inelastic scars that undergo slow and incomplete regression in comparison to normal scars. Pathophysiologically, HSc is characterized by excess collagen synthesis and deposition by fibroblasts in the wound (Boykin and Molnar, 1992; Scott et al, 1994). It is well established that cytokines are important regulators in wound healing and scar formation. Accumulating evidence indicates that heterogeneity of fibroblasts in the dermis may play a role in the formation of HSc, since fibroblasts derived from HSc tissue differ in collagen and collagenase production from those from normal skin (Ghahary et al, 1993; Ghahary et al, 1996; Tredget et al, 1993).

It is likely that NO plays a role in wound healing and scar formation, mainly through its vasodilation and antiproliferative effects (Benrath et al, 1995; Heck et al, 1992; Jorens et al, 1992). Adequate amounts of NO produced by endothelial cells, fibroblasts, and infiltrated leukocytes in tissue will maintain the appropriate tone of blood vessels and the appropriate rate of cell proliferation to accommodate the requirements of the healing wound. Fibroblasts are the major cell population in healing wounds and scar tissue; thus, these cells may be important in maintaining an adequate NO concentration in tissue. Previously, we demonstrated that fibroblasts derived from normal human skin express NOS enzymatic activities, messenger RNAs, and proteins in vitro and are capable of using the arginine-NOS pathway to synthesize NO (Wang et al, 1996). In this report, we provide evidence that NOS expression and NO production are reduced in fibroblasts derived from hypertrophic scar in comparison to those derived from normal skin.

4.2 MATERIALS AND METHODS

4.2.1 Clinical Specimens

Skin biopsies were obtained from 6 patients (5 males and 1 female, 3 to 32 years of age, 4 to 15 months post-burn, 14 to 80 per cent of total body surface thermal injury by second or third degree burns) who had suffered thermal injury and had been treated at the Firefighter's Burn Treatment Unit and outpatient burn clinic at the University of Alberta Hospital. After approval by the institutional ethics review board, informed consent was obtained from each patient who demonstrated extensive areas of hypertrophic scarring characterized by raised, erythematous, pruritic, thickened, and non-compliant scars confined to the site of injury. Under local anesthesia, biopsies were taken from areas of HSc using a 6-mm punch. A site-matched control biopsy was obtained from uninjured skin in each of the patients. The tissue samples were immediately placed in ice-cold supplemented Dulbecco's modified Eagle medium (DMEM) as described previously (Wang et al, 1996), and transported to the laboratory for processing. Half of each biopsy was used for explant culture and the other half was processed, embedded in paraffin, and sectioned.

4.2.2 Cell Culture

Dermal fibroblasts were isolated from the punch biopsies by the standard explant procedure described previously (Tredget et al, 1993). The fibroblasts were cultured in supplemented DMEM as described previously (Wang et al, 1996). After four passages, the resulting cell population consisted of more than 98% fibroblasts by the following criteria: morphology, growth characteristics, typical aging profile, and uniformly positive Thy-1 (Esterre et al, 1992) immunostaining (monoclonal antibody against human Thy-1; McKenzie and Fabre, 1981). Cell cultures at passages 5 to 9 were used in all experiments.

4.2.3 Spectrophotometric Estimation of NO Production

NO production by HSc and normal fibroblasts was assessed by measuring the nitrite accumulation in the culture media by a spectrophotometric method based on the Griess reaction (Green et al, 1990). The detailed procedure has been described previously (Wang et al, 1996). For positive controls, nitrite production by J774A.1 cells, a mouse monocyte/macrophage cell line from American Type Culture Collection, was measured in parallel (Fujihara et al, 1994).

To compare the NO production by different strains of fibroblasts, each pair of the HSc and normal fibroblasts derived from the same patient were cultured, treated, and examined in parallel using the above procedure. Six pairs of cells were examined. The measurement for each pair was repeated once.

4.2.4 Detection of Ca^{2+}-Dependent and -Independent NOS Activities

The citrulline assay, which is based on the measurement of the conversion of $[^{14}C]$L-arginine to $[^{14}C]$L-citrulline by NOS in the cytosol of the cells (Knowles et al, 1990; Schulz et al, 1992) was used. In brief, confluent fibroblasts were incubated in the absence (control) or presence of hrIFN-γ and LPS for 24 hrs. The cells were washed, trypsinized, and resuspended (2 x 10^7 cells/ml) in ice-cold N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (Knowles et al, 1990). The cell suspensions were homogenized, centrifuged (100,000 *g*; 35 min) at 4°C, and the cytosolic fraction was kept on ice for immediate assay. The membrane fraction was resuspended in HEPES buffer containing 0.01% Triton X-100 (Mallinckrodt Inc. Paris, KY) in a volume equal to original volume. For each sample, 20 µl of the cytosolic or membrane fraction was incubated in duplicate with 100 µl of assay buffer, pH 7.2, containing 50 µCi/ml L-[U-^{14}C]arginine (Amersham), 50 mM KH_2PO_4, (Knowles et al, 1990). After incubation at 37°C for 12 min, the samples were centrifuged at 10,000 *g* for 2 min. The [^{14}C]L-citrulline in the supernatant was separated from [^{14}C]L-arginine by cation-exchange chromotography with AG 50W-X8 resin (200-400 mesh; Bio-Rad, Richmond, CA) and quantified by liquid scintillation spectrometry in a Beckman LS6000IC scintillation counter. The protein content of the cytosol was determined by the Bradford dye-binding procedure (Bio-Rad) with bovine serum albumin as a standard.

The Ca^{2+}-dependent NOS activity was defined as the ethylene glycol-bis (beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)-inhibitabie conversion of L-[U-^{14}C]arginine to L-[U-^{14}C]citrulline. The Ca^{2+}-independent NOS activity was determined from the difference between the N^G-monomethyl-L-arginine (L-NMMA)-inhibitable and L-NMMA-EGTA-inhibitable NOS activities in the stimulated fibroblasts. For positive controls, rat brains and J774A.1 cells were analyzed for NOS activity in parallel with the HSc and normal fibroblasts (Wang et al, 1996).

4.2.5 Detection of mRNAs for endothelial cytosol and iNOS in HSc and Normal Fibroblasts Using RT-PCR

The total RNA was isolated from HSc and normal fibroblasts by a modified guanidinium isothiocyanate (GITC)/CsCl procedure of Chirgwin et al (1979). Five micrograms of the isolated RNA were run on a 1% agarose gel (GIBCO BRL) containing 1μg ethidium bromide per ml to confirm that there was no degradation during isolation. First-strand cDNA (RT-cDNA) was made from total RNA by using Superscript RNase H^- reverse transcriptase (Superscript RT I; GIBCO BRL) and Oligo $(dT)_{12-18}$ Primer (GIBCO BRL) as primers, using conventional procedures (Reiling et al, 1994).

The PCR was carried out in a RoboCycler® Gradient 40 temperature cycler (Stratagene Cloning Systems, La Jolla, CA). The primers and the PCR procedure have been described elsewhere (Wang et al, 1996). The amplified products were analyzed on a 1% agarose gel containing 1 μg ethidium bromide per ml. The expected sizes of the PCR products were determined by calculating the number of base pairs between the first nucleotide of the sense primer and the last nucleotide of the anti-sense primer complementary to corresponding human eNOS and hepatocyte iNOS cDNA sequences. The approximate sizes were determined by using 100 bp DNA molecular weight markers (GIBCO BRL). For positive controls, the plasmid cDNAs specific for human eNOS (Marsden et al, 1992) and hepatocyte iNOS (Geller et al, 1993) were used as templates in PCR.

For negative controls, total cellular RNA from normal and HSc fibroblasts without reverse transcription was used.

4.2.6 Detection of eNOS and iNOS Proteins in HSc and Normal Fibroblasts and Tissues Using Immunocytochemistry

A mouse peroxidase anti-peroxidase (Sigma) immunostaining procedure was used to detect eNOS and iNOS in HSc and normal fibroblasts. The detailed procedures for the immunostaining of eNOS and iNOS have been described elsewhere (Wang et al, 1996). To examine the source of NOS protein expression in HSc and normal tissue, 5 μm paraffin sections prepared from HSc and normal skin biopsies were stained with anti-human eNOS and anti-mouse macrophage iNOS monoclonal antibodies (Transduction Laboratories, Lexington, KY). Slides were examined and microphotographs were taken with a Nikon OPTIPHOT-2 microscope equipped with a FX-35DX camera (Nikon Optical, Tokyo, Japan).

4.2.7 Detection of eNOS and iNOS Expression in HSc and Normal Fibroblasts Using Fluorescent Activated Cell Sorting Analysis

To quantify NOS expression in cultured fibroblasts, we used FACS analysis to examine eNOS and iNOS proteins in HSc and normal cells. Indirect immunofluorescent staining was performed using the procedure of Reiling and colleagues (1994). In brief, confluent fibroblasts in culture flasks were treated with hrIFN-γ and LPS for 24 hrs. The stimulated and control cells were harvested by trypsinization, washed with PBS, and fixed with 4% paraformaldehyde. The cells were made permeable by incubating with 0.05% Tween-20 in PBS at room temperature for 30 min. Non-specific binding was blocked with 10% normal goat serum at room temperature for 2 hrs. The cells were then incubated with the primary antibodies (Transduction Laboratories, 5 μg/ml in PBS containing 0.2% BSA) for 18 hrs at 4°C. In negative controls, the primary antibodies were replaced with non-immune normal mouse IgG. After washing with PBS, the cells were incubated with FITC-conjugated goat-anti-mouse IgG (Sigma) at room temperature for 45 min. Labeled cells were washed with PBS and analyzed in a FACScan cytofluorometer using LYSYS II software (Becton Dickinson, San Jose, CA).

4.3 RESULTS

4.3.1 Both HSc and Normal Fibroblasts Spontaneously Produce NO, Which Is Increased by IFN-γ and LPS

Hypertrophic scar and normal fibroblasts were stimulated with hrIFN-γ and LPS alone or in combination for 96 hrs. Without any stimulation, both HSc and normal fibroblasts produced NO, but the HSc cells produced significantly lower amounts than normal fibroblasts ($p < 0.01$, $n = 6$, paired t test). Neither IFN-γ nor LPS alone significantly stimulated the production of NO; however, addition of IFN-γ and LPS together significantly increased the NO production by both HSc and normal fibroblasts (**Figure IV-1**). Both HSc and normal fibroblasts showed concentration-dependent responses to hrIFN-γ and LPS (data not shown). The combination of 200 U of hrIFN-γ per ml and 40 μg of LPS per ml for a period of 96 hrs induced maximum production of nitrite by the cells and was used in all subsequent experiments (Wang et al, 1996).

Six pairs of dermal fibroblast strains derived from 6 individuals were examined for NO production. Without stimulation, all strains of HSc fibroblasts produced less NO than their counterpart normal fibroblasts (**Figure IV-2A**). After stimulation, 5 out of 6 of the HSc cell strains produced significantly lower levels of NO than their counterpart normal cells (**Figure IV-2B**). All strains of stimulated HSc and 5 out of 6 strains of normal fibroblasts produced significantly higher levels of NO than the unstimulated normal cells (**Figure IV-2A** and **-2B**). Overall, the unstimulated HSc cells produced significantly lower levels of NO than unstimulated normal cells (7.37 ± 1.17 vs 11.15 ± 0.79 nmol/10^6 cells/96 h; $p < 0.01$, $n = 6$, paired t test), as did the stimulated HSc cells (15.70 ± 1.34 vs 21.99 ± 2.16 nmol/10^6 cells/96 h; $p < 0.01$, $n = 6$, paired t test). The proportionate increase after stimulation was similar, however, for HSc (47% increase) and normal (51% increase) cells. For positive controls, stimulated and unstimulated J774A.1 cells, a mouse monocyte/ macrophage cell line, were used (data not shown).

4.3.2 HSc Fibroblasts Express Less Ca^{2+}-Dependent NOS Activity Than Normal Fibroblasts

In pilot experiments we found that NOS activities appeared only in the cytosolic fraction of human fibroblasts, so the membrane suspensions were not examined in subsequent experiments. **Table IV-1** shows the Ca^{2+}-dependent and -independent NOS activities from cytosolic extracts of rat brain, J774A.1 cells, HSc, and normal fibroblasts as measured by the citrulline assay. Rat brain and J774A.1 cells were used as positive controls for determining Ca^{2+}-dependent and -independent NOS activities, respectively, because they are well-known to express eNOS and iNOS, respectively (Bredt et al, 1992; Knowles et al, 1990). The unstimulated HSc fibroblasts expressed significantly less Ca^{2+}-dependent NOS activity than unstimulated normal fibroblasts (**Table IV-1**). After stimulation, the

HSc fibroblasts also expressed significantly less Ca^{2+}-dependent NOS activity than did normal cells, but the Ca^{2+}-independent NOS activity in stimulated HSc fibroblasts was similar to that in stimulated normal cells (**Table IV-1**).

4.3.3 HSc and Normal Fibroblasts Express mRNAs for both eNOS and iNOS

Since both Ca^{2+}-dependent and -independent NOS activities are observed in HSc and normal fibroblasts, we examined the expression of mRNAs for eNOS (Marsden et al, 1993) and hepatocyte iNOS (Geller et al, 1993) in the cells using RT-PCR. **Figures IV-3A** and **-3B** show the ethidium bromide-stained PCR products for eNOS and iNOS in agarose gels respectively. Lanes 1 in Figure 3A and B show amplification of eNOS and iNOS mRNA derived cDNAs using the corresponding plasmid cDNAs for positive controls. The 422 bp product for eNOS and the 462 bp product for hepatocyte iNOS are the correct sizes for these products as indicated by the DNA ladder (lanes M in **Figure IV-3A** and **-3B**). When the total cellular RNAs without reverse transcription were used, the bands for eNOS and iNOS were not apparent (lanes 2 in **Figure IV-3A** and **-3B**). The results showed that both normal (lanes 3 to 5) and HSc (lanes 6 to 8) fibroblasts contained mRNAs for eNOS (panel A) and iNOS (panel B).

We also examined the mRNA for eNOS in stimulated HSc and normal cells as well as the mRNA for hepatocyte iNOS in unstimulated HSc and normal fibroblasts using RT-PCR. The result showed that the eNOS mRNA was detectable in all strains of stimulated HSc and normal fibroblasts. The bands of RT-PCR products from stimulated HSc and normal fibroblasts showed density similar to those from unstimulated cells in ethidium bromide-stained agarose gel (data not shown). The mRNA for hepatocyte iNOS was not detectable in unstimulated HSc and normal fibroblasts.

4.3.4 Hypertrophic Scar Fibroblasts Express Both eNOS and iNOS Proteins

Like normal fibroblasts (Wang et al, 1996), almost all of the HSc fibroblasts appeared to be stained with the anti-eNOS antibody under low magnification, but some cells showed intense staining (data not shown). Under high magnification, the cytoplasm around the nuclei of the cells had stronger staining in some intensely stained fibroblasts but the staining in other cells was scattered throughout in the cytoplasm.

The unstimulated control cells and stimulated control cells did not show staining when the primary antibody was replaced with non-immune mouse IgG. The stimulated HSc fibroblasts showed staining similar to that of normal cells when stained with iNOS antibody. When the primary antibody was replaced with non-immune mouse IgG, the stimulated control cells and unstimulated control cells did not stain (data for the HSc cells not shown; for the normal cells, see Wang et al, 1996).

4.3.5 Epidermal Keratinocytes and Dermal Fibroblast-like Cells Express eNOS

After immunostaining normal skin for eNOS, most keratinocytes in the epidermis, some of the fibroblast-like cells (arrow), and blood vessels (not shown) were positive (**Figure IV-4a**). The keratinocytes located at the middle part of the epidermis showed very strong staining (**Figure IV-4a**). In HSc tissue, some of the epidermal keratinocytes stained for eNOS, but the staining was much weaker than in normal skin although they were processed on a single slide using the same procedure (**Figure IV-4b**). The fibroblast-like cells in the dermis also showed weaker staining than those in normal dermis (data not shown). Both normal and HSc tissue did not stain for iNOS (**Figure IV-4c** and **-4d**). When the primary antibodies were replaced by non-immune mouse IgG, very little or no staining was present in the cells in normal and HSc tissue (**Figure IV-4e** and **-4f**).

4.3.6 Hypertrophic Scar Fibroblasts Express Less eNOS Protein As Determined by Fluorescent Activated Cell Sorting

Fluorescent activated cell sorting (FACS) analysis showed that the HSc and normal fibroblasts expressed eNOS without stimulation. Although all normal and HSc fibroblasts were positive after immuno-fluorescent staining, the fluorescence intensity of HSc cells was lower than that of normal fibroblasts (**Figure IV-5A**). After stimulation, both HSc and normal fibroblasts expressed iNOS (**Figure IV-5B**). Analysis of FACS data with the LYSYS II software indicated that 32% of the stimulated normal cells were positive when the threshold was set at 5% for the control in which the primary antibody was replaced by non-immune mouse IgG. Only 19% of the stimulated HSc fibroblasts were positive at the same threshold (data not shown). The fluorescence intensity of the HSc cells, however, was similar to that of normal fibroblasts after immunofluorescent staining for iNOS (**Figure IV-5B**).

4.4 DISCUSSION

The physiological functions and the pathophysiological roles of NO in the circulatory, neuronal, and immune systems have been well-established (Moncada et al, 1991; Nathan, 1992). In addition, NO has been shown to have anti-neoplastic (Hibbs et al, 1988; Stuehr and Nathan, 1989) and anti-proliferative (Firnhaber and Murphy, 1993; Garg and Hassid, 1990) effects and to play a critical role in septic shock (Forstermann et al, 1993; Zembowicz and Vane, 1992). Many types of mammalian cells are able to synthesize NO through the arginine-NOS pathway (Nathan, 1992). Previously, we demonstrated that human dermal fibroblasts synthesized NO and expressed both eNOS and iNOS (Wang et al, 1996). In this study, we provide evidence that fibroblasts derived from HSc tissue express less NOS and produce lower levels of NO than fibroblasts derived from normal cells obtained from the same patient.

Both HSc and normal fibroblasts constitutively synthesized NO in vitro, but the HSc fibroblasts produced significantly lower levels of NO than normal cells. This is a general phenomenon, as all 6 pairs of fibroblasts derived from 6 individuals showed a similar result. This was further confirmed by the radioactive citrulline assay, which examined the enzyme activity of eNOS in the cytosol of the cells. The results of FACS analysis also showed decreased expression of eNOS protein in the HSc fibroblasts. These results collectively suggested that the post-burn HSc fibroblasts may be defective to some degree in expression of eNOS, as a result of a phenotypic change leading to the down-regulation of eNOS expression. This resembles the decreased collagenase mRNA and enzyme activity in HSc cells as compared to their normal counterparts (Ghahary et al, 1996). This reduction of collagenase mRNA and enzyme activity may be a consequence of the down-regulated eNOS expression, since NO has been shown directly to activate collagenase activity in human articular cartilage cells. Both NOS and collagenase can be induced by inflammatory cytokines and LPS and can be inhibited by competitive inhibitor of NOS (Murrell et al, 1995). This suggests another possible regulatory mechanism for NO or peroxynitrite in fibrotic disorders of tissue.

After stimulation of the cells with hrIFN-γ and LPS, both HSc and normal fibroblasts produced significantly higher levels of NO. The proportional increase was similar for the HSc and normal cells indicating that the HSc cells retained the capability to express iNOS. The significance of iNOS expression in fibroblasts is not clear. It can be speculated that in the case of skin injury with bacterial infection, LPS and cytokines released by infiltrated leukocytes stimulate the dermal fibroblasts and infiltrated leukocytes to express iNOS. This higher level of NO might play a role in maintaining the blood supply to the wound site and in controlling bacterial infection during the early stages of wound healing. Moreover, the slightly higher NO levels inside and adjacent to the fibroblasts might protect the cells from damage caused by oxygen free radicals released by activated infiltrating leukocytes such as neutrophils and macrophages, since NO can neutralize both oxygen and hydroxyl radicals (Harbrecht et al, 1992). Like the other human cell types studied so far, such as monocytes/macrophages, the dermal fibroblasts produced much less NO than rodent cells after stimulation (Fujihara et al, 1994).

It is of interest that after stimulating the cells with hrIFN-γ and LPS, human fibroblasts expressed more Ca^{2+}-dependent activity as determined by citrulline assay.

Since HSc and normal fibroblasts produced low levels of NO and expressed low Ca^{2+}-dependent and -independent iNOS activities, we used RT-PCR to detect the mRNAs for eNOS and iNOS in unstimulated and stimulated cells. We assume that the eNOS and iNOS expressed in human fibroblasts are identical to those found in human monocytes/macrophages. Because of the nature of PCR and the difficulty of controlling the efficiency of reverse transcription, it is difficult to accurately quantify gene expression using RT-PCR without a well-designed and well-established internal standard. Thus, quantification of the eNOS and iNOS mRNA expression by HSc and normal fibroblasts was not performed in this study.

The immunocytochemical results showed that like the normal fibroblasts (Wang et al, 1996), the unstimulated HSc cells express eNOS in vitro. The pattern of staining was also similar between the HSc and normal cells: all cells were stained; some showing intense staining, especially in the cytoplasm close to the nucleus. After stimulation, some of the HSc fibroblasts, like normal cells (Wang et al, 1996), stained for iNOS. Furthermore, immunohistochemical results also showed that HSc tissue expressed less eNOS that normal skin, although this technique is not quantitative. Interestingly, most of the epidermal keratinocytes were stained for eNOS. This result is consistent with the recent report indicating that normal human keratinocytes express eNOS in vitro (Baudouin and Tachon, 1996). The keratinocytes in HSc tissue express less eNOS than those in normal skin. This seems not to be an artifact, because the normal and HSc tissues were processed on a single slide and stained simultaneously. These results also support the assumption that NO plays a role in wound healing and scar formation (Heck et al, 1992).

Data from FACS analysis showed that fibroblasts derived from HSc tissue and normal skin expressed eNOS and iNOS. Although all stained for eNOS, the fluorescence intensity of the HSc cells was lower than that of normal cells. This result suggests that all of the HSc fibroblasts retain the ability to express eNOS, but that the amount is down-regulated. This defective eNOS expression might be a consequence of the altered cytokine production by the cells, since cytokines such as transforming growth factor beta (TGF-β) have been shown to suppress NO production of murine macrophages (Vodovotz et al, 1993). We have demonstrated that HSc tissue expresses more TGF-β mRNA and protein than normal skin (Ghahary et al, 1993; 1995). This suggests a possible regulatory pathway, in which TGF-β down-regulates the expression of eNOS. This low level of endogenous NO could cause over-proliferation of fibroblasts, resulting in HSc formation. After stimulation of the cells with IFN-γ and LPS, the proportion of cells positive for iNOS seems lower in HSc fibroblasts than in normal cells, but the HSc and normal fibroblasts showed similar fluorescence intensity. Moreover, the fluorescence intensity was much lower than that for eNOS, suggesting that iNOS expression in human fibroblasts was very low. Thus, a small increase in cells staining for iNOS seems insufficient to cause a statistically significant increase in the Ca^{2+}-independent NOS activity as assessed by citrulline assay.

The significance of the reduced NO production in HSc fibroblasts is not clear but it is possible that after thermal injury, the fibroblasts in the scar of some patients undergo phenotypic alteration, leading to reduced eNOS expression. The low levels of endogenous NO in the fibroblasts, along with other factors, may allow increased proliferation and over-production of certain fibrogenic cytokines such as transforming growth factor-β and IL-1, resulting in HSc formation. On the other hand, HSc fibroblasts might be deficient in co-factors such as tetrahydrobiopterin (Werner-Felmayer et al, 1990) or substrates such as L-arginine for the enzyme. Supplementation of deficient co-factors or substrate might increase NO production and result in reduced proliferation and production of fibrogenic cytokines by the cells.

In conclusion, dermal fibroblasts derived from HSc contain less eNOS and produce less NO than normal fibroblasts. The expression of iNOS is not altered in HSc fibroblasts. These data suggest that NO may play a role in the cellularity of wound healing and HSc formation.

Table IV-1. **Origin and Characteristic of Human Skin Biopsies from 6 Individuals**

Patient #	Age (years)	Sex	Biopsy site[a]	TBSA[b] (%)	Post-burn (months)
1	18	M	Hand	80	7
2	28	F	Chest	14	15
3	13	M	Neck	35	5
4	4	M	Shoulder	25	6
5	32	M	Chest	60	5
6	3	M	Leg	28	4

[a] The biopsy sites for HSc are indicated and the biopsies of normal skin were taken from the nearest available uninjured area.

[b] Total body surface area that was injured by second or third degree burns in the original thermal injury.

Table IV-2. **HSc Fibroblasts Express Less eNOS Activity Than Normal Cells**

		NOS ACTIVITY(pmol/mg/min)[a]	
		eNOS (Ca^{2+}-Dependent)	iNOS (Ca^{2+}-Independent)
NORMAL FIBROBLASTS	Stimulated	2.60 ± 0.54	1.59 ± 0.14
	Unstimulated	1.36 ± 0.57	N. D.[b]
HSc FIBROBLASTS	Stimulated	1.43 ± 0.19[c]	1.27 ± 0.10
	Unstimulated	0.63 ± 0.20[c]	N. D.
J774A.1 CELLS	Stimulated	9.67 ± 2.52	19.05 ± 3.96
	Unstimulated	2.27 ± 0.83[d]	N. D.
RAT BRAIN	(n = 4)	2.86 ± 0.29	N. D.

[a] Determined using the radioactive citrulline assay as described in "Materials and Methods" and expressed as pmol of L-arginine converted per mg of protein in the cytosol per min (pmol/mg/min ± SEM).

[b] N. D.: not detectable.

[c] Statistically significant when compared with their counterpart normal cells ($p < 0.05$, n=4).

[d] Statistically significant when compared with unstimulated cells ($p < 0.01$, n=4).

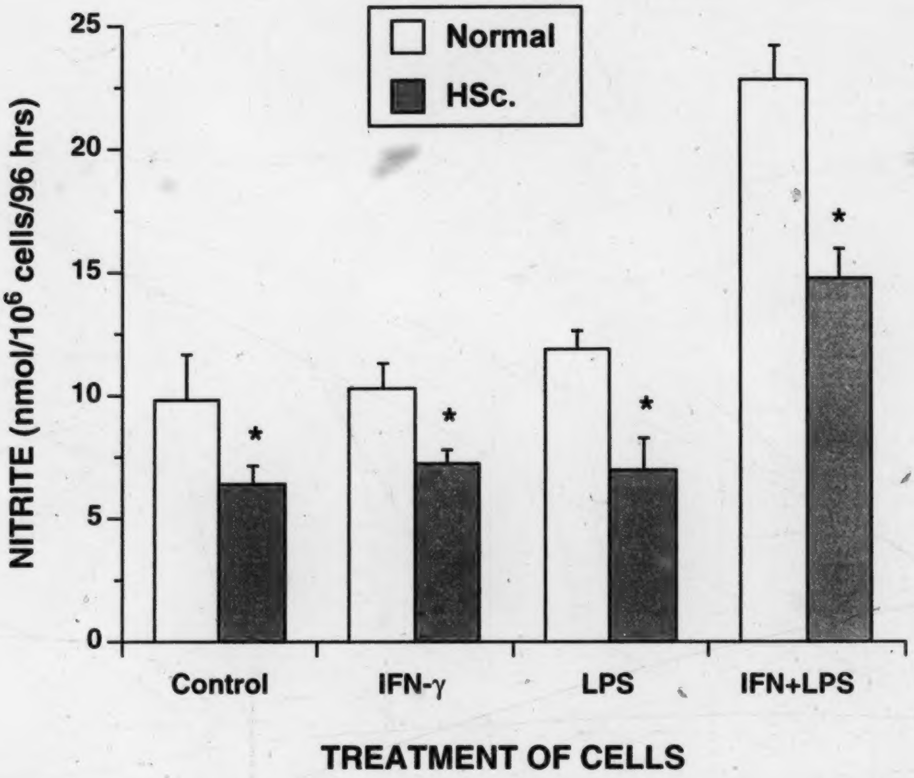

Figure IV-1. **Normal and HSc fibroblasts spontaneously produce NO, which is increased after stimulation:** Confluent normal and HSc fibroblasts in 24-well plates were treated with PBS (control), hrIFN-γ alone, LPS alone or hrIFN-γ plus LPS for 96 hrs. Nitrite in the cell-free supernatants was measured using the procedures described in "Materials and Methods". Data represent mean ± SEM obtained from 3 separate experiments. Asterisks (*) indicate the significant difference ($p < 0.05$) between normal and HSc fibroblasts. The significant difference between the stimulated fibroblasts and their corresponding controls was also observed in both normal and HSc cells.

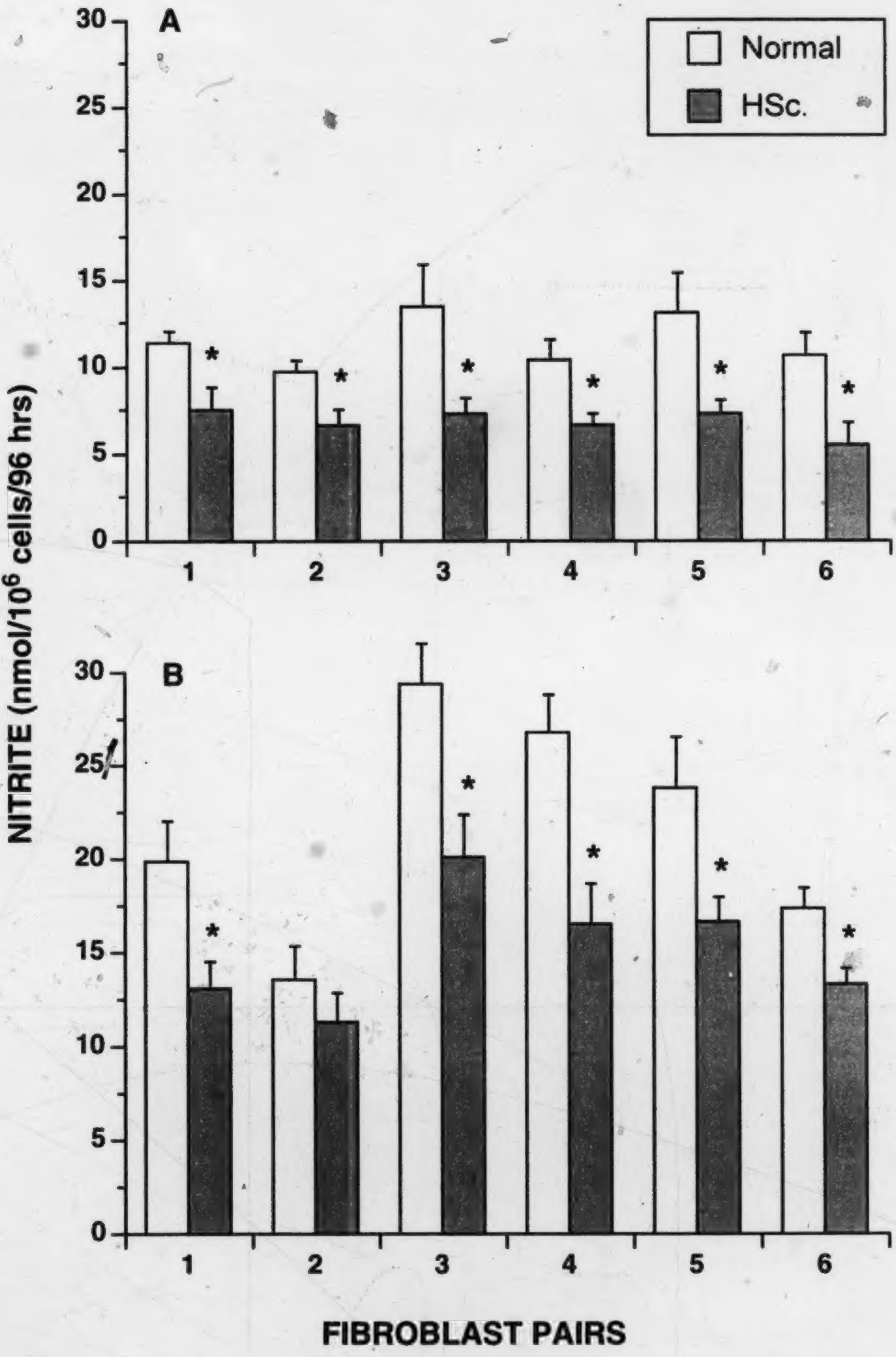

Figure IV-2. See legend on next page.

Figure IV-2. **HSc cells produce less NO than their counterpart normal cells:** (*A*), *NO production by HSc and normal fibroblasts without stimulation*. Both HSc and normal fibroblasts in 24-well plates were incubated for 96 hrs. Nitrite in the supernatants were measured with the Griess reaction. (*B*), *NO production by HSc and normal fibroblasts after stimulation*. The HSc and normal fibroblasts were stimulated with hrIFN-γ and LPS for 96 hrs. Nitrite accumulation in the supernatant was measured with the Griess reaction. Data for A and B represent mean ± SEM obtained from 3 separate experiments for each pair of cells. Asterisks (*) indicate a significant difference ($p < 0.05$; n=3; ANOVA) between normal and HSc cells with or without stimulation.

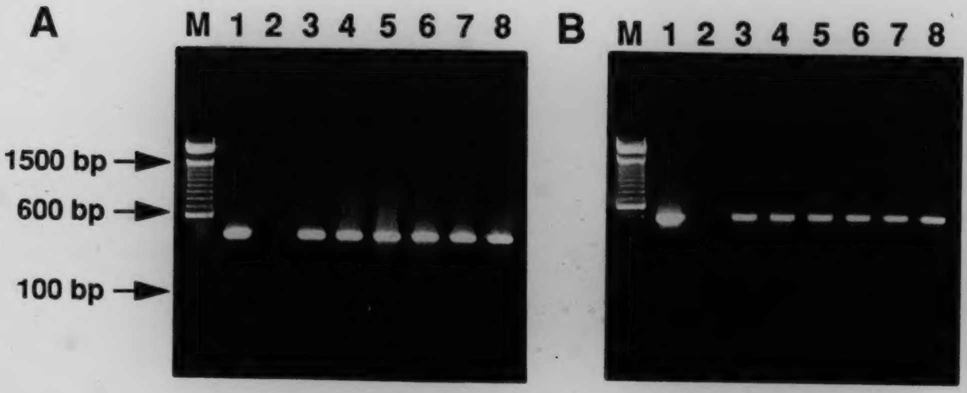

Figure IV-3. **HSc and normal fibroblasts express eNOS and iNOS mRNAs:** RT-PCR was used to detect mRNAs for eNOS (panel A) and iNOS (panel B). Photograph represents the eNOS and iNOS PCR products stained by ethidium bromide. Lanes M show the 100 bp DNA ladder. Lanes 1 in panel A and B show positive controls using plasmid cDNA for eNOS and iNOS, respectively. Lanes 2 in panel A and B show the negative controls using RNA without reverse transcription for eNOS and iNOS. Lanes 3 to 5 in Figure 3A show the PCR products for eNOS using RT-cDNA from 3 strains of normal fibroblasts. Lanes 6 to 8 in Figure 3A show the PCR products for eNOS from 3 strains of HSc cells. Lanes 3 to 5 and 6 to 8 in Figure 3B show the PCR products for iNOS using RT-cDNA from the corresponding strains of fibroblasts stimulated with hrIFN and LPS.

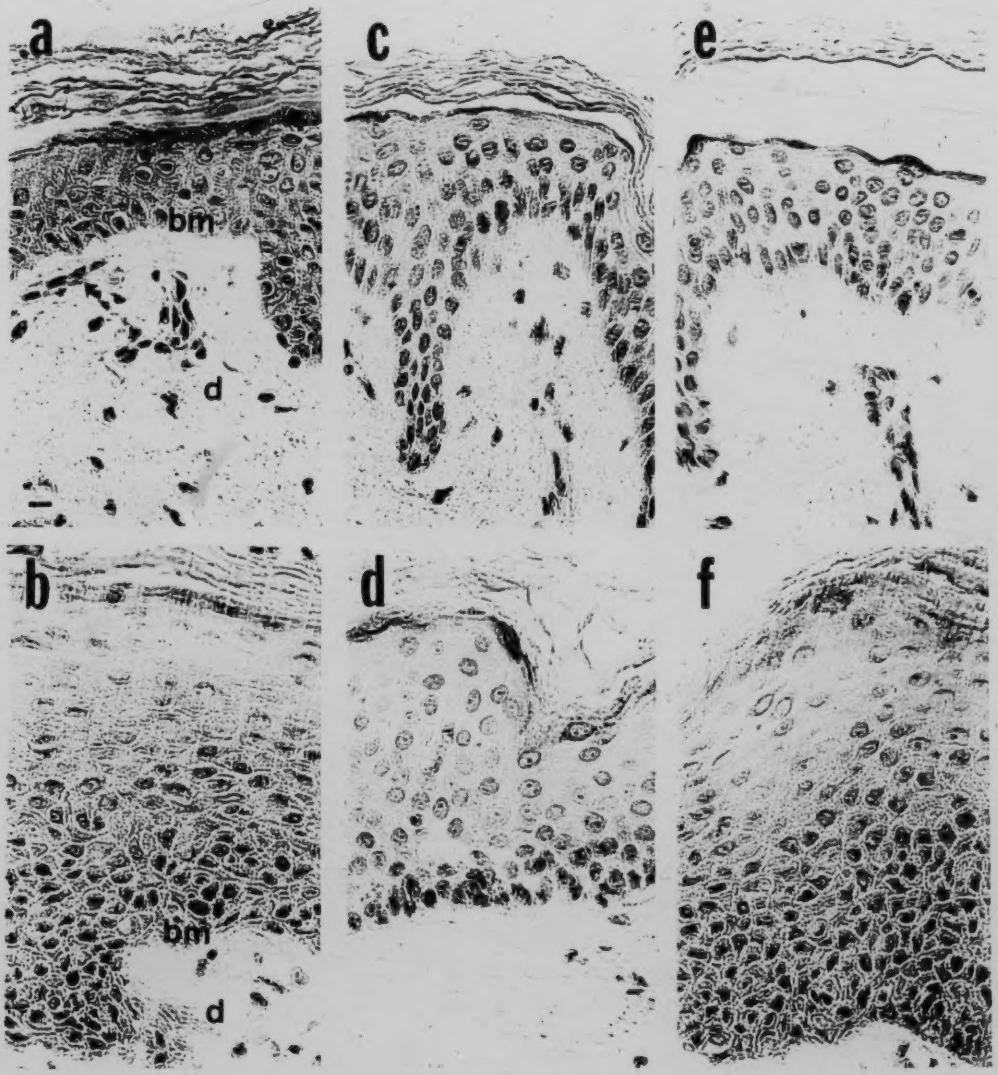

Figure IV-4. See legend on next page.

Figure IV-4. **Expression of eNOS proteins is lower in HSc tissues relative to normal skin obtained from the same patient:** Paraffin-embedded sections from HSc and normal skin punch biopsies were stained with the peroxidase anti-peroxidase procedure to visualize eNOS and iNOS proteins. Panels (a) and (c) show normal skin stained for eNOS and iNOS, respectively. Panels (b) and (d) show the HSc tissue stained for eNOS and iNOS, respectively. Panels (e) and (f) are negative controls for normal skin and HSc, in which the primary antibodies were replaced by non-immune mouse IgG. (*a*), *View of normal skin after immunostaining for eNOS*. Most keratinocytes in the epidermis and some fibroblasts (arrow) in the dermis (**d**) showed positive staining. The bar indicates 2.5 µm for all panels. **bm**: basement membrane. (*b*), V*iew of HSc tissue after immunostaining for eNOS*. Some of the keratinocytes in the epidermis were stained with the eNOS antibody. Some of the fibroblast-like cells in the dermis (d) showed very faint staining with the eNOS antibody. (*c*) and (*d*), V*iew of normal skin and HSc tissue after iNOS immunostaining*. None of the keratinocytes in the epidermis or the fibroblast-like cells in the dermis showed staining with the iNOS antibody. The brown dots in the basement membrane area are artifacts. (*e*), *and* (*f*), V*iew of negative controls for normal skin and HSc tissue*. All the cells in the epidermis and dermis are negative.

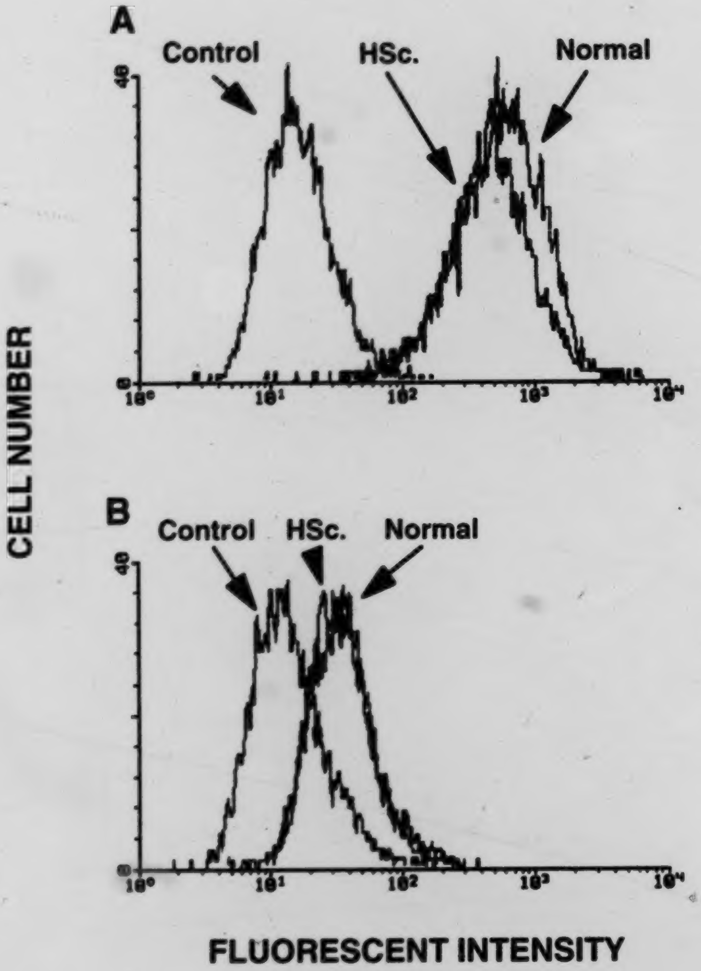

Figure IV-5. **HSc cells express less eNOS than normal fibroblasts as determined by FACS analysis:** HSc and normal fibroblasts were stained with an indirect immunofluorescent staining procedure as described in "Materials and Methods" and subjected to FACS analysis. Two pairs of HSc and normal cell strains have been examined. Panels A and B show the histogram data of FACS analysis for eNOS and iNOS, respectively, in one pair of the cell strains.

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CHAPTER FIVE

HYPERTROPHIC SCAR TISSUES AND FIBROBLASTS PRODUCE MORE TGF-β1 mRNA AND PROTEIN THAN NORMAL SKIN AND CELLS: ASSAYED BY QUANTITATIVE RT-PCR AND ELISA[*]

5.1 INTRODUCTION

Hypertrophic scarring (HSc) is a fibroproliferative disorder of the dermis characterized by raised, red, nodular and inelastic scars that undergo slow and incomplete regression in comparison to normal scars. HSc frequently occurs after wounding, particularly after severe thermal injury of the skin (Scott et al, 1994). Pathophysiologically, HSc is characterized by excess collagen synthesis and deposition by fibroblasts in the wound (Scott et al, 1994). It is well established that cytokines are important regulators in wound healing and scar formation. Accumulating evidence indicates that heterogeneity of fibroblasts in the dermis may play a role in the formation of HSc, since fibroblasts derived from HSc tissue differ in collagen and collagenase production from those from normal skin (Ghahary et al, 1996; Tredget et al, 1993).

Transforming growth factor-β1 (TGF-β1) is a multi-functional cytokine produced by a variety of cells (Roberts et al. 1990). This cytokine plays a major role in tissue fibrosis via its fibrogenic activity on fibroblasts (Roberts et al, 1986).

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TGF-β1 protein is more abundant in tissues of fibrotic skin disorders such as HSc in comparison to normal skin (Ghahary et al, 1995). Fibroblasts have been demonstrated to express TGF-β1 protein both in vitro and in vivo (Ghahary et al, 1995).

Development of the reverse transcription-polymerase chain reaction (RT-PCR) has ushered in a new era in the study of gene expression, particularly in detection of low-abundance messages in small amounts of tissue or cells. This powerful tool makes the study of short-lived, low-copy number, mRNA transcripts possible (Wang et al, 1989). Quantitative RT-PCR is now a widely accepted technique for detection and quantitation of specific messages (Kanangat et al, 1992). Many strategies have been used to generate internal standards for quantitative RT-PCR. One approach uses relatively invariant mRNA such as β-actin (Uberla et al, 1991), β2-microglobulin (Murphy et al, 1990), glyceraldehyde-3-phosphate dehydrogenase (Sharefkin et al, 1991), or an unrelated template as an endogenous internal standard (Rappolee et al, 1988). The disadvantage of this approach is that it only provides relative or comparative data (Wang et al, 1989). The other strategies include the use of subcloned genomic DNA with a small intron (Gilliland et al, 1990), a synthetic DNA fragment formed by oligonucleotide overlap extension and subsequent PCR (Ho et al, 1989; Dillon and Rosen, 1990), or a DNA fragment containing a specific restriction site created by site-directed mutagenesis using PCR (Perrin and Gilliland, 1990). These strategies usually involve extensive DNA synthesis, poly-A tailing, or multiple subcloning, which require considerable development and standardization (Nicoletti and Sassy-Prigent, 1996). An alternative quantitative RT-PCR using a mathematical approach has been reported recently (Nicoletti and Sassy-Prigent, 1996). This approach uses mathematical calculation of the relative amount of mRNA after serial dilution of the sample and a reference molecule followed by RT-PCR. However, this approach can only provide, once again, comparative data. It is necessary to develop a simple, reliable procedure to generate internal standards for RT-PCR, which can be used in an individual laboratory as a routine protocol to quantify a gene of interest in a minute amount of tissue from patients. The purpose of this study is to establish a quantitative RT-PCR procedure for detection of TGF-β1 gene expression in skin biopsies under normal conditions and in the diseased situation after treatment. For this purpose, we established a convenient, reliable, single subcloning procedure to construct internal standards for competitive RT-PCR starting from a gene specific PCR product for human TGF-β1. This procedure is useful in generating gene specific internal standards for any DNA (genomic or cDNA) or mRNA, provided that the sequences for the target genes are known.

5.2 MATERIALS AND METHODS

5.2.1 Clinical Specimens

Skin biopsies were obtained from patients who had developed HSc characterized by raised, erythematous, pruritic, thickened, and non-compliant scars confined to the site of thermal injury and had been treated at the Firefighter's Burn Treatment Unit and outpatient burn clinic at the University of Alberta Hospital. After approval by the institutional ethics review board, informed consent was obtained from each patient. Under local anesthesia, biopsies were taken from areas of HSc using a 6-mm punch. A control biopsy was obtained from nearby uninjured skin in each of the patients. The tissue samples were immediately placed in ice-cold supplemented Dulbecco's modified Eagle's medium as described previously (Wang et al, 1996), and transported to the laboratory for processing. Half of each biopsy was used for explant culture and the other half was processed, embedded in paraffin, and sectioned for other studies.

5.2.2 Cell Culture

Dermal fibroblasts were isolated from the punch biopsies by the standard explant procedure described previously (Tredget et al, 1993). The fibroblasts were cultured in supplemented Dulbecco's modified Eagle's medium. For isolation of total cellular RNA, HSc and normal fibroblasts were sub-cultured in 100 mm tissue culture dishes (Corning). When the cells reached 70 to 80 per cent confluence, the medium in the cultures was replaced with fresh medium containing 2% fetal bovine serum. The dishes were incubated for an additional 72 hr under normal culture conditions and the cells were harvested for extraction of total cellular RNA. For measurement of TGF-β1 protein, HSc and normal fibroblasts were subcultured in 6-well plates (Corning) in a density of 150,000 cells per well in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. After 24 hr, the medium was replaced with fresh medium containing 2% fetal bovine serum. The plates were incubated for an additional 96 hr under normal culture conditions and the medium in each well was harvested individually for measurement of TGF-β1 protein by ELISA.

5.2.3 Isolation of Total RNA from Cells and Tissues

Total RNA was isolated from cultured dermal fibroblasts and from HSc and normal skin using the procedure of Chomczynski and Sacchi (1987). Briefly, the cells in tissue culture dishes were washed with ice-cold PBS 3 times and lysed with 500 μl of Solution D (Chomczynski and Sacchi, 1987). HSc and normal tissues were weighed on an electronic scale and placed into 1.5 ml microcentrifuge tubes containing 500 μl of Solution D. The tissues were minced and homogenized using a Teflon tissue homogenizer. The cell lysates and tissue suspensions were extracted with phenol : chloroform : isoamyl alcohol (25 : 24 : 1). The resulting solutions were precipitated with one volume of isopropanol and 0.1 volume of 2 M sodium acetate (Chomczynski and Sacchi, 1987). The RNA pellets were redissolved in 20 μl of TE buffer (pH 8) and the RNA concentrations were determined by spectrophotometry. Five micrograms of the isolated RNA were run on a 1% denaturing agarose gel (GIBCO BRL) containing 1μg ethidium bromide per ml to confirm that there had been no degradation during isolation.

5.2.4 Construction of Internal Standards and Generation of cRNA for Human TGF-β1

To construct the internal standards for TGF-β1, a 63 bp cDNA fragment was obtained from an endonuclease Mae II digestion of human procollagen α1(I) cDNA (American Type Culture Collection, 12301-Parklawn Drive, Rockville, MD) by electrophoresis separation and elution. A 442 bp PCR product corresponding to nucleotides 1158-1600 of human TGF-β1 cDNA was digested with Mae II to generate 2 fragments that contain compatible ends with the 63 bp fragment. After dephosphorylation, these 2 fragments were ligated with the 63 bp fragment to generate a 505 bp construct, which was then re-amplified with PCR. A PCR cloning kit from Stratagene Cloning Systems (La Jolla, CA) was used to subclone the 505 bp DNA fragment into pCR-Script SK^+ plasmid. This vector was linearized with an unique endonuclease Srf I, which was also present in the ligation reaction to prevent direct religation of the plasmid. After insertion of the target DNA fragment, the site for Srf I was destroyed, thus a very high percentage of positive recombinant plasmid was obtained. The reconstructed plasmid, designated pCR-TGF-β1, was then amplified in *E. coli* XL1-Blue MRF' Kan. The amplified plasmid was sequenced using an automated fluorescent labeling DNA sequencer and primers complementary to bacteriophage T7 and T3 promoters to confirm the orientation of the insert.

To generate the human TGF-β1 specific complementary RNA (cRNA) internal standard, plasmid pCR-TGFβ1 was linearized with Not I and transcribed *in vitro* using an RNA synthesis kit (Promega, Madison, WI). Sense cRNA was synthesized using T7 RNA polymerase and purified by phenol-chloroform extraction and sodium acetate-ethanol precipitation for three times. The concentration of the cRNA was determined by measuring the optical absorbency at 260 nm using an UV spectrophotometer. The cRNA, designated internal standard cRNA (IS-cRNA), was stored at -20°C at a concentration of 50 ng/μl in 75% ethanol containing 200 mM sodium acetate.

5.2.5 Competitive Reverse Transcription-Polymerase Chain Reaction

First-strand cDNA (RT-cDNA) was made from 1 μg of total cellular RNA and varying amounts of IS-cRNA (6 tubes, each tube containing 1 μg of total RNA plus 3.125, 6.25, 12.5, 25, 50, or 100 ng of IS-cRNA) by using Superscript RNase H^- reverse transcriptase (Superscript RT-I; Gibco BRL) and sequence specific antisense primer (see below) for human TGF-β1 (conventional procedures from Superscript RT-I data sheet, Gibco BRL). The RT-cDNA for tissue RNA was prepared from total tissue RNA equivalent to 400 μg of tissue and varying amounts of IS-cRNA as used for cellular RNA.

The PCR was carried out in a RoboCycler® Gradient 40 temperature cycler (Stratagene Cloning Systems, La Jolla, CA). The following gene-specific primers were used to amplify the desired cDNA: TGF-β1 sense (5'-CAA GCA GAG TAC ACA CAG CA-3') and TGF-β1 antisense (5'-GAT GCT GGG CCC TCT CCA GC-3'), which correspond to nucleotides 1158-1178 and 1580-1600 of the human TGF-β1 cDNA, respectively (Tarnuzzer et al, 1996). The primers were synthesized in the Nucleic Acid Research Center, University of Alberta. The reaction mixture contained reaction buffer with 2.5 mM $MgCl_2$, 100 μM of each of the dATP, dCTP, dGTP and dTTP, 25 pmol of sense and antisense primers, 2.5 units Taq DNA polymerase (GIBCO BRL) and 1/5 of the RT-cDNA in a final volume of 50 μl. The PCR conditions were 75 seconds of denaturing at 97°C, 75 seconds of annealing at 54°C, and 30 seconds of extension at 73°C for 35 cycles with an initial denaturing cycle at 97°C for 3 min and a final extension cycle at 72°C for 5 min. The amplified products were analyzed on a 1.2% agarose (Gibco BRL) gel containing 1 μg/ml ethidium bromide. The sizes of the PCR products were calculated from the number of base pairs between the sense and anti-sense primers aligned with the corresponding human TGF-β1 cDNA sequences and indicated by the 100 bp DNA molecular weight markers (GIBCO BRL). For positive controls, the plasmid cDNA specific for human TGF-β1 was used as template in PCR. For negative controls, 200 ng of total cellular RNA plus 20 ng of IS-cRNA without reverse transcription was used.

5.2.6 Quantitation of RT-PCR Products

The images of the RT-PCR products in agarose gels were recorded by using a Polaroid camera under ultraviolet illumination. The specific bands for internal standards and TGF-β1 mRNA were scanned with an Apple OneScanner connected to a Macintosh IIci computer and digitized with the NIH Image v1.59 program. The results for the internal standards and TGF-β1 mRNA were plotted against the amount of IS-cRNA used in reverse transcription to generate IS-cDNA for PCR and the amount of RT-cDNA for TGF-β1 was calculated. The amount of TGF-β1 mRNA per µg of total cellular RNA was calculated based on the numbers of molecules of internal standards in each PCR reaction and corrected with the molecular weight of internal standards and the molecular weight of the plasmid. The number of copies for TGF-β1 mRNA per cell was calculated based on the assumption that a nucleated mammalian cell expresses 10 pg of total RNA (Kanangat et al, 1992).

5.2.7 Northern Analysis of TGF-β1 mRNA in HSc and Normal Fibroblasts

Ten micrograms of total cellular RNA was separated by electrophoresis and blotted onto nitrocellulose membranes. The membranes were baked under vacuum for 2 h at 80°C and pre-hybridized in a pre-hybridization solution for 4-6 h at 42°C, as described (Ghahary et al, 1993). Hybridization was performed in the same solution at 42°C for 16-20 hrs using cDNA probes for human TGF-β1, or 18 S ribosomal RNA. The cDNA probe for TGF-β1 was obtained from Dr. G. I. Bell (Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biology and Medicine, University of Chicago, Chicago, IL). The probe for 18 S ribosomal RNA was obtained from American Type Culture Collection (Rockville, MD) and used to demonstrate that apparent differences in TGF-β1 mRNA levels in HSc and normal cells did not result from variations in loading. The probes were labeled with ^{32}P-α-dCTP (Du Pont Canada, Mississauga, Ontario, Canada) by nick-translation. After hybridization the membranes were initially washed at room temperature with 2 x sodium chloride: sodium citrate buffer (1 x sodium chloride: sodium citrate buffer = 150 mM sodium chloride and 15 mM sodium citrate) and 0.1% sodium dodecyl sulphate for 30 min, then for 20 min at 65°C in 0.1 x sodium chloride: sodium citrate buffer and 0.1% sodium dodecyl sulphate solution. Autoradiography was performed by exposing Kodak X-Omat film to the nitrocellulose membrane at -80°C in the presence of an enhancing screen.

5.2.8 ELISA for TGF-β

To determine the amounts of TGF-β protein in the supernatants from fibroblast cultures, we used a sandwich ELISA based on the procedure reported by Danielpour et al (1989). Briefly, 96-well ELISA plates were coated with 100 μl per well of monoclonal antibody against human TGF-β (anti-human TGF-β1, 2, 3; Genzyme, Cambridge MA) at a concentration of 1 μg/ml in PBS. The plates were incubated for 2 hr at room temperature followed by 16 hr at 4°C. After washing twice with PBS-Tween-20 (PBS-T), the plates were blocked with 1% bovine serum albumin (BSA, Sigma) for 60 min at room temperature and washed 3 times with PBS-T. The serum samples were extracted using an acid-alcohol extraction procedure (Danielpour et al, 1993). The culture media were acidified with 24 μl per ml of 5N HCl for 15 min at room temperature and neutralized with 40 μl per ml of 1 M HEPES/5 N NaOH (5/2, v/v). One hundred microlitres per well of the extracted serum or acidified/neutralized medium samples were added to the wells of the plates, which were then incubated at room temperature for 60 min. After washing with PBS-T 3 times, the plates were incubated with 100 μl per well of chicken anti-human TGF-β (R & D Systems Inc. Minneapolis, MN) at a concentration of 5 μg/ml for 60 min at room temperature with shaking. After washing 5 times with PBS-T, the plates were incubated with alkaline phosphatase-conjugated rabbit anti-chicken IgG (Sigma) at room temperature for 60 min followed by washing with PBS-T 5 times. After addition of the substrate (o-nitrophenyl phosphate, 1 mg/ml, Sigma), the plates were incubated at room temperature for 60 min and the optical density was read using a THERMO max (Molecular Devices, Menlo Park, CA) microplate reader at wavelength of 405 nm. Serial dilutions (0 to 2500 pg/ml) of recombinant human TGF-β (Genzyme) were used as standards in each plate to quantify TGF-β in the samples.

5.3 RESULTS

5.3.1 The Recombinant Plasmid Contains Human TGF-β1 Sequence, as Shown by Endonuclease Digestion, PCR Amplification, and DNA Sequencing

Figure V-1 shows the procedure for constructing the internal standard. After digestion with Pvu II, plasmids from 7 colonies of *E. coli* XL1-Blue MRF Kan transformed with pCR-TGF-β1 yielded a fragment of the expected size (953 bp made up 505 bp TGF-β1 sequence plus 448 bp plasmid sequence). Plasmids from 6 out of 7 colonies were positive for TGF-β1 after PCR amplification. The expected 505 bp TGF-β1 PCR product was obtained in 5 of the 6 positive plasmids. DNA sequence using bacteriophage T7 and T3 promoter sequence specific primers showed that the 505 bp TGF-β1 sequence was present in 1 (the only one sent for sequencing) of the positive plasmids. The orientation of TGF-β1 cDNA in the plasmid was such that the T7 promoter sequence preceded the sense strand and the T3 promoter sequence preceded the anti-sense strand.

5.3.2 The Synthetic TGF-β1 cRNA and Cellular RNA can be Reverse Transcribed and Amplified at a Similar Efficiency

After in vitro transcription of the plasmid with bacterophage T7 RNA polymerase, the resulting cRNA contained the human TGF-β1 sequence as determined by PCR amplification and restriction endonuclease digestion of the PCR products (data not shown). **Figure V-2** shows the products of co-amplification of synthetic and cellular cDNA after reverse transcription. The results showed that the efficiency of reverse transcription and PCR amplification of both synthetic and cellular cRNA was similar over a wide range from 0.01 to 100 ng of synthetic cRNA and 10 to 1000 ng of cellular RNA per reaction. The linear range was about 0.1 to 50 ng of synthetic cRNA and 50 to 500 ng of cellular RNA. Thus, 0.625 to 20 ng of synthetic cRNA, corresponding to 1.6×10^9 to 2.6×10^{10} molecules per reaction, was used in subsequent experiments to quantify TGF-β1 mRNA in cells and tissues.

5.3.3 HSc Fibroblasts Express more mRNA for TGF-β1 than Normal Cells

Expression of TGF-β1 mRNA in 5 pairs of HSc and normal fibroblasts was compared. **Figure V-3A** shows the RT-PCR products for synthetic and cellular TGF-β1 for one of the 5 pairs of dermal fibroblasts examined. The bands in ethidium bromide-stained agarose gel were scanned and digitized and the results plotted against the amount of synthetic TGF-β1 template in the internal standard (**Figure V-3B**). The equivalence point for internal standard and cellular RNA was obtained and used to calculate the number of TGF-β1 molecules in the reaction on a per cell basis. **Figure V-3C** shows the results for 5 pairs of HSc and normal cells. On average, dermal fibroblasts from HSc expressed 116 ± 6 while normal cells expressed 97 ± 7 molecules of TGF-β1 transcripts per cell ($p < 0.01$, $n = 5$, paired t test).

To verify the competitive RT-PCR results, Northern analysis was used to quantify TGF-β1 mRNA in HSc and normal fibroblasts. The results indicated that dermal fibroblasts from HSc tissues produced significantly more TGF-β1 mRNA than normal cells and the differences were maintained over passages 3 to 5 (**Figure V-4A** and **-4B**).

5.3.4 HSc Tissues Contains more TGF-β1 mRNA than Normal Skin

To compare mRNA for TGF-β1 in HSc tissue and in normal skin, total RNA from those tissues was extracted and examined with the competitive RT-PCR. **Figure V-5A** shows the PCR products in an ethidium bromide-stained agarose gel. For calculation purposes, 3-times more total RNA from normal skin was used in the competitive RT-PCR to raise the amounts of template to the detection range of the internal standards as shown in **Figure V-5B**. The calculation indicated that HSc tissue contained 5.7 ± 1.3 fold more TGF-β1 mRNA than normal skin.

5.3.5 HSc Fibroblasts Produce more TGF-β Protein than Normal Cells

Supernatant from HSc and normal fibroblast cultures was examined for TGF-β protein using a sandwich ELISA. Five pairs of HSc and normal cells were examined. All 5 strains of HSc fibroblasts produced more TGF-β protein than their counterpart normal cells (**Figure V-6**). Overall, HSc cells produced 1.62 ± 0.26 pg while normal cells produced 1.36 ± 0.28 pg of TGF-β proteins per 1000 cells per 96 hrs ($p < 0.01$, $n = 5$, paired t test).

5.4 DISCUSSION

Post-burn HSc is a serious clinical problem for plastic surgery and rehabilitative medicine. It frequently causes severe cosmetic and functional impairment in burn patients. The etiology of HSc and mechanism of HSc formation are not clear. Many factors such as bacterial infection, delayed epithelization, and genetic factors are considered responsible for HSc formation (Scott, et al. 1994; Tredget et al, 1997). Fibroblasts play a major role in wound healing and scar formation. The products of fibroblasts such as collagens, metalloproteinases, and cytokines are important in wound healing and scar remodeling (Scott, et al. 1994; Tredget et al, 1997). Phenotypic alterations in collagen synthesis, collagenase production (Ghahary, et al. 1995) and nitric oxide synthase expression (Wang et al. 1997) have been reported in fibroblasts derived from HSc tissue. These alterations may be the consequence of altered cytokine production by the cells. To examine this possibility, it is important to know that some important fibrogenic cytokines such as TGF-β1 produced by the cells are altered.

TGF-β1 is a multifunctional cytokine produced by platelets, macrophages, lymphocytes, fibroblasts and many other types of cell. It functions as an anti-proliferative agent for cells of ectodermal lineage while stimulating proliferation of cells of mesenchymal lineage such as fibroblasts (Sporn and Roberts, 1987). TGF-β1 is considered a fibrogenic cytokine since it promotes collagen synthesis and deposition by fibroblasts, enhances wound healing and increases wound breaking strength (Beck et al. 1993). Confirmation of the correlation between TGF-β1 production and HSc may provide evidence that TGF-β1 is involved in HSc formation.

The present paper describes a new strategy to generate gene specific cRNAs as exogenous internal standards for competitive RT-PCR to quantify mRNA expression in small amounts of tissue or a small number of cells. The cRNA generation protocol is simple, reproducible, and only requires a single cloning process. An important advantage of this protocol is that no radioactive or neurotoxic materials such as acrylamide are involved in the procedure of construction of cRNA and analysis of PCR products. The construction procedure starts with a gene specific PCR or RT-PCR product, which can be generated using existing primers. The initial step is to identify an unique restriction site in the PCR product and insert a 50 to 100 bp DNA fragment with the same restriction sites. The recombinant molecule is significantly different in size from the wild-type molecules permitting easy separation of their PCR products by agarose gel electrophoresis. This direct analysis of PCR products eliminates any additional manipulation of the amplified cDNA, thus eliminating many factors that may affect the quantification. The synthetic cRNA and cellular RNA are reverse transcribed and amplified in the same tube, thus all variables related to efficiency of reverse transcription and PCR amplification are internally controlled. The use of a gene-specific primer rather than oligo d(T) primer in reverse transcription has the distinct advantage that it eliminates the possible difference in efficiency of reverse-transcription between cellular poly-(A) RNA, which consists of multiple species of mRNAs, and synthetic poly-(A) RNA, which is composed of only gene-specific RNA. The only drawback to using a gene-specific primer in reverse transcription is that different primers are required for different genes.

Another advantage of this approach is that the sequences of the synthetic template and the target molecule differ very little. This minimizes possible differences in efficiency of reverse transcription and PCR amplification due to different nucleotide composition of the synthetic templates and the target molecules. The small difference in length of the synthetic and target molecules should have little effect on the amplification efficiency because of the nucleotide incorporation capacity of thermal stable DNA polymerase (> 60 nucleotides per second; Kanangat et al, 1992). This approach eliminates the differences of primer efficiency and reverse transcription efficiency, tube variation, and variations in reaction conditions. However, some of the problems inherent in PCR, such as substrate saturation and product inhibition of the enzyme, incomplete strand separation, strand reannealing, and sensitivity to molecular contamination (Kozbor et al, 1993), remain. These require careful optimization of the RT-PCR conditions for individual primer-template pairs.

PCR has been considered to be the most powerful tool for mRNA and DNA detection (Wang et al, 1989). Theoretically, a single DNA molecule or mRNA copy could be detected by PCR or RT-PCR (Erlich et al, 1991). However, due to many technical limitations, less than 10,000 copies of a DNA sequence would barely give positive result even with the more sensitive procedure using radioactive primers (Kanangat et al, 1992).

We used this approach to quantify TGF-β1 mRNA in fibroblasts derived from HSc and normal skin. All 5 strains of HSc cells produced more TGF-β1 mRNA than their counterpart normal cells. An average of 16% more TGF-β1 mRNA was observed in HSc cells. Northern analysis confirmed that HSc cells produced more TGF-β1 mRNA. At the protein level, HSc cells produced an overall 15% more TGF-β protein than normal cells as measured by ELISA. These results indicate that quantitative RT-PCR is a valid procedure for mRNA quantitation in this context.

The final goal of developing this procedure was to quantify mRNA for TGF-β and other extracellular matrix proteins in skin biopsies from HSc patients after various treatments. In a preliminary experiment, we compared TGF-β1 mRNA expression in HSc and normal skin. The results showed that HSc tissue contained much more TGF-β1 mRNA than normal skin. The greater difference seen in the tissue samples compared to cultured fibroblasts might come from the cellularity of HSc tissue, in which fibroblasts are more abundant than in normal skin (Ghahary et al, 1995; Tredget et al, unpublished observation). The infiltrated leukocytes, especially macrophages, may also be responsible for the increased TGF-β mRNA in the tissue (Cavaillon, 1994). Furthermore, fibroblasts in HSc tissue could be under the influence of TGF-β stimulating factors such as hypoxia (Falanga et al, 1991). The RT-PCR data for TGF-β1 mRNA from cultured fibroblasts only reflects the difference between HSc and normal fibroblasts.

It is well-known that TGF-β1 induces its own expression (Obberghen-Schilling et al, 1988; Kim et al, 1990). The autoinduction of TGF-β1 is reported to occur through its classical regulatory pathway, the AP-1 complex, which binds to the 5'-flanking regions of TGF-β1 gene to initiate expression (Kim et al, 1989). It is reasonable to assume that TGF-β1 released by activated platelets and infiltrated leukocytes stimulates the autoinduction of TGF-β1 by fibroblasts in the wound. The higher level of TGF-β1 produced by fibroblasts in the wound would enhance collagen synthesis and inhibits collagen degradation, resulting in development of HSc.

In conclusion, a simple, reproducible procedure to construct a gene-specific template for competitive RT-PCR to quantify human TGF-β1 mRNA expression has been developed. Data from RT-PCR and Northern analysis revealed that HSc fibroblasts and tissue contained more TGF-β1 mRNA than normal cells and tissue.

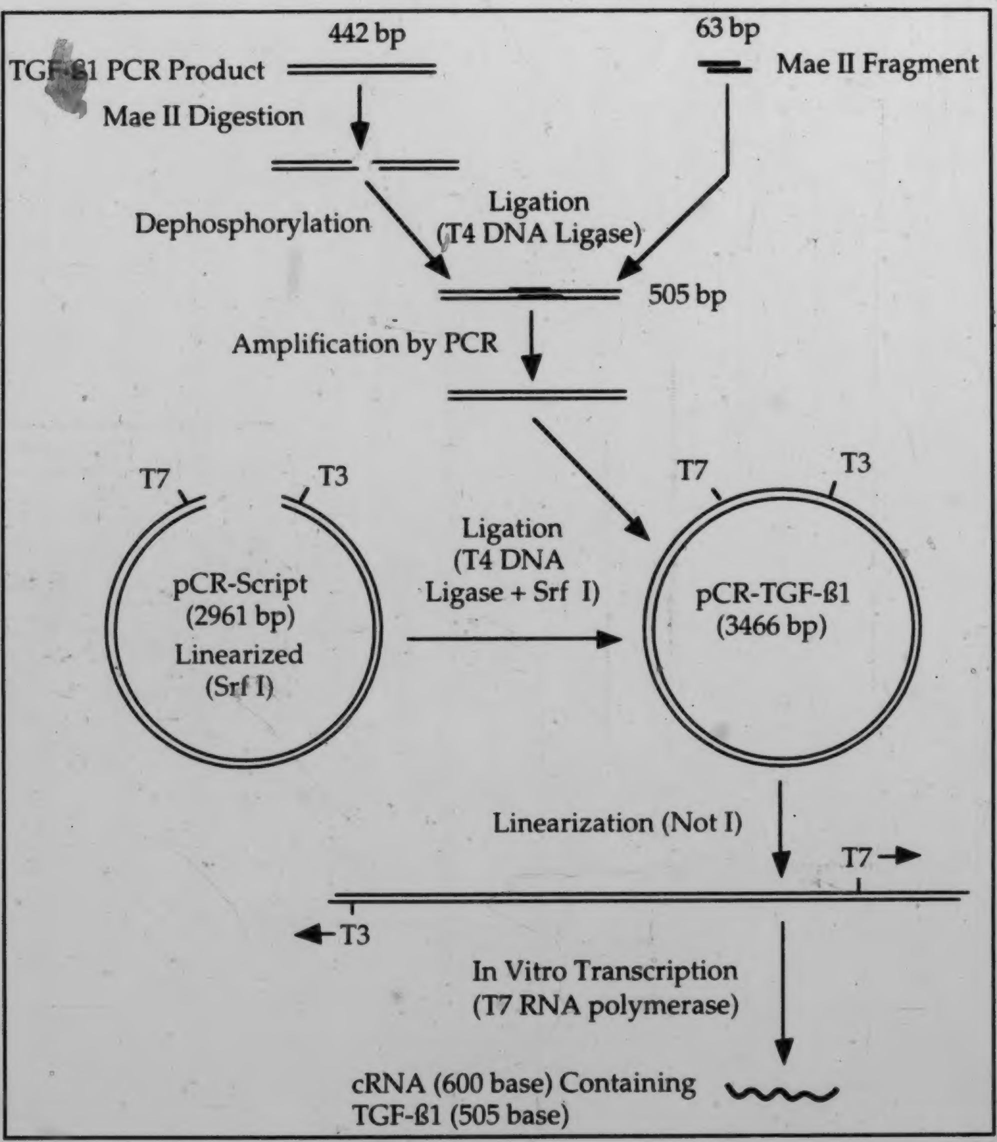

Figure V-1. See legand on next page.

Figure V-1. **Construction of Gene Specific Internal Standard for Competitive RT-PCR to Quantify Human TGF-β1 mRNA:** The 442 bp PCR product was obtained by amplification of a plasmid containing 2.1 kb human TGF-β1 cDNA using gene specific primers described in the text. An unique Mae II site, located at nucleotide 124 downstream in the sense strand, was identified using the DNA analysis program DNA Strider v1.2. After digestion with Mae II, the DNA fragments were dephosphorylated by bovine intestine phosphatase to prevent re-ligation. A 63 bp Mae II fragment, identified and isolated from a plasmid containing human procollagen α1 (I) cDNA, was inserted into the digested PCR fragments. The recombinant fragment was then re-amplified by PCR using the same primers. This new 505 bp PCR product was cloned into plasmid pCR-Script™ and amplified in bacteria using a PCR cloning kit. The recombinant plasmid was then linearized with Not I and transcribed in vitro with T7 RNA polymerase. The resulting cRNA was a 600 base fragment containing the 505 base TGF-β1 sequence, which was used in reverse transcription after phenol : chloroform extraction and ethanol precipitation.

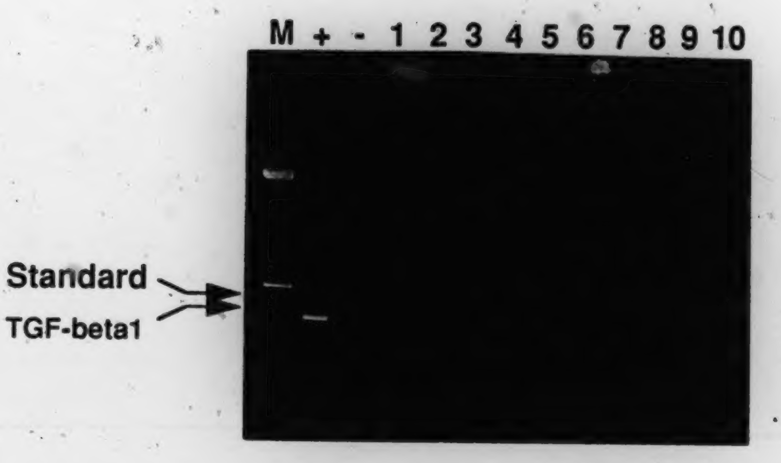

Figure V-2. **Co-amplification of Synthetic and Cellular cDNAs with PCR after Reverse Transcription:** One hundred nanograms of synthetic cRNA and 1 μg of cellular RNA were serially diluted and reverse transcribed. One fifth of the volume in each reaction was amplified with PCR. The products were separated on an 1.2% agarose gel containing 1 μg per ml of ethidium bromide. Lane M is the 100 bp DNA marker. Lane "+" is the positive control using plasmid cDNA containing human TGF-β1 sequence. Lane "-" is the negative control in which 20 ng of synthetic cRNA and 50 ng of cellular RNA without reverse transcription were used. Lanes 1 to 10 show the RT-PCR products generated from serially diluted synthetic cRNA and cellular RNA (0.02 to 20 ng of synthetic cRNA and 0.2 to 200 ng of cellular RNA).

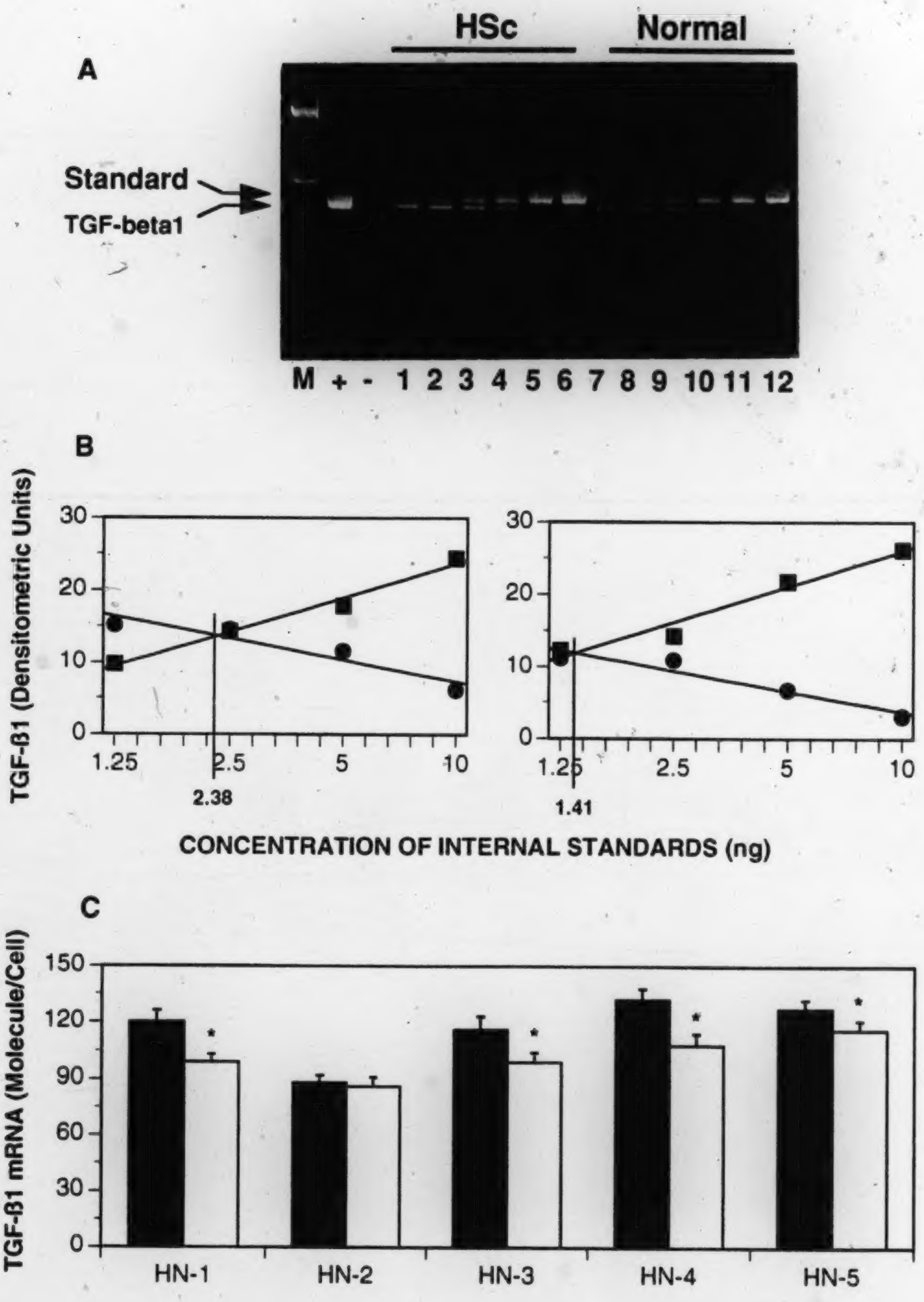

Figure V-3. See legend on next page.

Figure V-3. **TGF-β1 mRNA Expression in 5 Pairs of HSc and Normal Fibroblasts:** Five pairs of dermal fibroblasts derived from HSc and normal skin were examined for TGF-β1 mRNA expression. Panel A: RT-PCR products for TGF-β1 mRNA in one of the 5 pairs of cells. Lane M: 100 bp DNA marker; Lane "+": positive control using human TGF-β1 plasmid cDNA; Lane "-": negative control using synthetic cRNA and cellular RNA without reverse transcription. Lane 1 to 6: different amounts of synthetic cRNA (0.625 to 20 ng) plus 200 ng of cellular RNA from HSc cells; Lane 7 to 12: different amounts of synthetic cRNA (0.625 to 20 ng) plus 200 ng of cellular RNA from normal fibroblasts. Panel B: Plots of the densitometric data derived from Panel A. For clarity, only the densitometric data from Lanes 2 to 5 for HSc and Lanes 8 to 11 for normal cells are shown. The amount of TGF-β1 mRNA used in reverse transcription was determined and the number of TGF-β1 mRNA molecules per cell was calculated as described in "Material and Methods" (—■—: internal standards; —●—: cellular RNA). Panel C: TGF-β1 mRNA expression in 5 pairs of HSc and normal cells. Total cellular RNA from 5 pairs of HSc and normal fibroblasts was quantified and expressed as number of TGF-β1 mRNA molecules per cell. Each column indicates the mean of two duplicate experiments (± SEM). Asterisks indicate significant differences between HSc (closed column) and normal (open column).

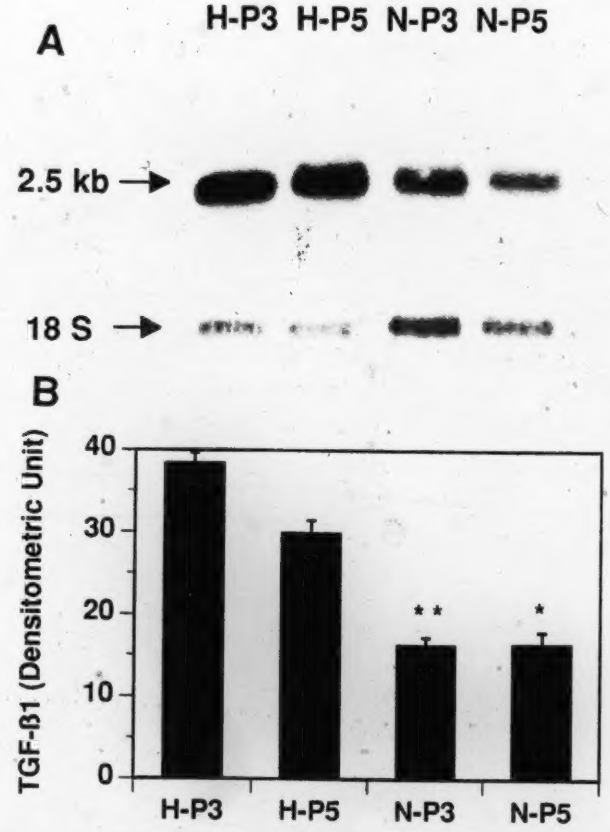

Figure V-4. **Northern Analysis of TGF-β1 mRNA in HSc and Normal Fibroblasts:** Ten micrograms of total cellular RNA from HSc and normal cells were separated in an 1% agarose gel, transferred onto a nitrocellulose membrane, and blotted with ^{32}P-labelled cDNA probes specific for human TGF-β1 and 18 S ribosomal RNA as loading control. Panel A shows the autoradiography of one of the three pairs of HSc and normal cells. P3 and P5 indicate the cell passage number 3 and 5, respectively. The bands for the 2.5 kb TGF-β1 transcript were scanned, digitized and plotted (Panel B). The bands for 18 S ribosomal RNA were used to correct for loading. Asterisks indicate significant differences between HSc and normal cells in TGF-β1 mRNA expression (*: $p < 0.05$, **: $p < 0.01$, $n = 3$, student t test).

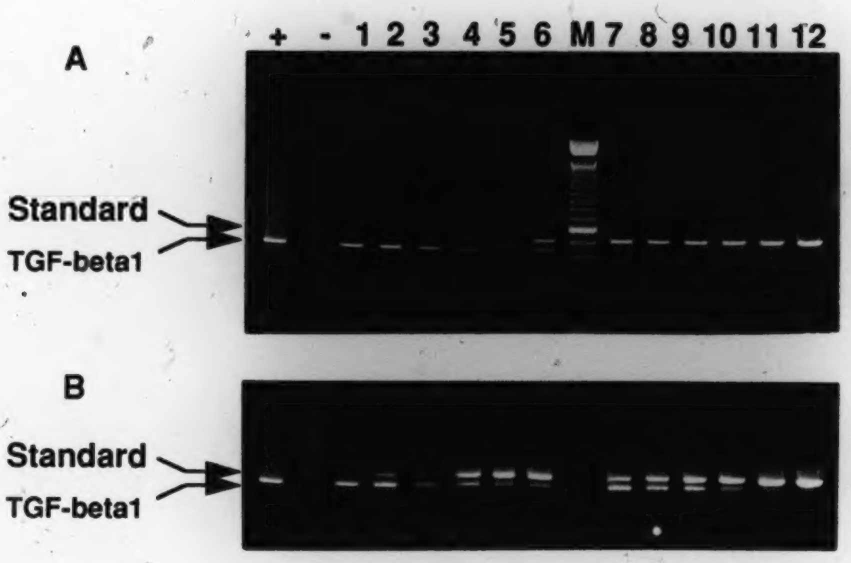

Figure V-5. See legend on next page.

Figure V-5. **TGF-β1 mRNA Expression in HSc Tissue and Normal Skin:** Total RNA was isolated from HSc and normal tissue, reverse transcribed along with synthetic cRNA and subjected to PCR. Panel A shows the RT-PCR products using total RNA from 400 μg of HSc or normal tissue and various amounts of internal standard cRNA in an ethidium bromide stained agarose gel. Panel B shows RT-PCR products using total RNA from 400 μg of HSc or 1.2 mg of normal skin and various amounts of internal standard cRNA. Lane M: 100 bp DNA marker; Lane "+": positive controls using plasmid containing human TGF-β1; Lane "-": negative controls using synthetic cRNA and cellular RNA without reverse transcription; Lanes 1 to 6: total RNA from HSc plus synthetic cRNA (0.625 to 20 ng); Lanes 7 to 12: total RNA from normal skin plus synthetic cRNA (0.625 to 20 ng).

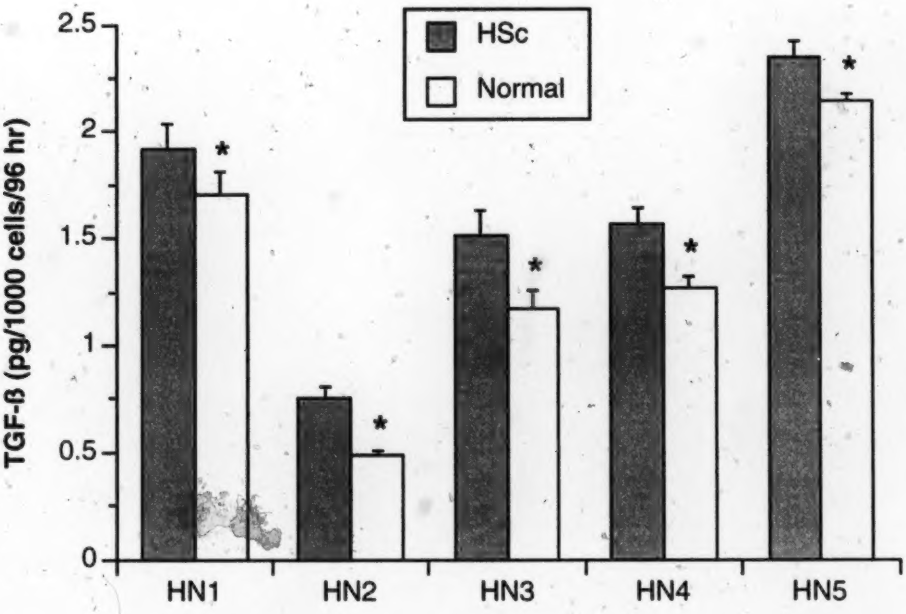

Figure V-6. **Detection of TGF-β Protein in the Supernatant of HSc and Normal Cell Cultures by ELISA:** TGF-β protein in the cell culture supernatant were examined with a sandwich ELISA. HSc and normal fibroblasts were cultured in 6-well plates. Medium from triplicate wells was collected and assayed for TGF-β after acidification and neutralization. The cells were harvested and counted with a Coulter Counter. The amount of TGF-β was expressed as pg per 1000 cells per 96 hrs. Asterisks indicate significant differences between HSc and normal cells ($p < 0.05$ for all 5 pairs, triplicate experiments, paired t test). The experiment was repeated twice.

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CHAPTER SIX

TRANSFORMING GROWTH FACTOR-β mRNA AND PROTEIN IN HYPERTROPHIC SCAR FIBROBLASTS AND TISSUES: ANTAGONISM BY INTERFERON α/γ IN VITRO AND IN VIVO*

6.1 INTRODUCTION

Hypertrophic scarring (HSc) is a dermal fibroproliferative disorder that frequently occurs following damage to the deep dermis of the skin by thermal injury and other forms of trauma. It often causes severe cosmetic and functional impairment, especially in young patients (Scott et al, 1994; Tredget et al, 1997). Transforming growth factor-β1 (TGF-β1) belongs to the type beta TGF class of a superfamily of functionally and structurally related cytokines (Sporn and Roberts, 1990). It is a fibrogenic growth factor produced by a variety of cells including fibroblasts. TGF-β1 has been shown to stimulate mesenchymal cells to proliferate and produce more extracellular matrix proteins (Roberts et al, 1990). We have demonstrated that mRNA and immunoreactive protein for TGF-β are increased in the nodular regions of HSc (Ghahary et al, 1994). Interferons (IFNs) are a family of cytokines with antiviral and immunoregulatory activities. Two types of IFNs have been identified based on their sequence and receptor binding properties. They are produced by all nucleated cells and have a variety of biological activities. Besides their antiviral and immunoregulatory properties, IFNs have been reported to inhibit the proliferation and extracellular matrix protein production by fibroblasts and mesangial cells. Both type I and type II IFNs have been employed to treat fibroproliferative disorders such as hepatic cirrhosis, keloid and rheumatoid arthritis (Castilla et al., 1991; Granstein et al, 1990; Hein et al, 1992; Larrabee et al, 1990).

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In previous work, we have compared HSc fibroblasts to pair-matched normal dermal fibroblasts from the same patients and found that HSc cells produce excessive matrix proteins such as type I and type III procollagens and fibronectin (Ghahary et al, 1993), less collagenase (Ghahary et al, 1995), decorin (Scott et al, 1995; 1996) and nitric oxide (Wang et al, 1996; 1997). Treatment of HSc fibroblasts with IFN-γ (Harrop et al, 1995) or -α2b (Tredget et al, 1993) reduces procollagen mRNA and collagen protein synthesis and cell proliferation. Interferon-α2b but not -γ increases collagenase mRNA, leading to increased collagenase enzyme activity in vitro (Ghahary et al, 1995).

Recently, systemic elevations of TGF-β1 in vivo have been described in animal models of autoimmune systemic lupus erythematosus (Caver et al, 1996), in breast cancer patients who develop liver and pulmonary fibrosis after bone marrow replacement and chemotherapy (Anscher et al, 1993) and in burn patients who develop severe hypertrophic scarring (Tredget et al, in press). Suppression of the elevated serum TGF-β1 in burn patients after systemic treatment with IFN-α2b was associated with normalization of both TGF-β1 and plasma histamine. Because it is known that TGF-β1 can auto-induce its own production through AP-1 complex in the 5'-flanking region of the TGF-β gene (Kim et al 1990), investigation of the production of TGF-β1 by fibroblasts from HSc was conducted to see if these cells are capable of TGF-β1 production which could contribute to the elevated serum levels observed in burn patients. Many of the features of the activated HSc fibroblast characterized above resemble the normal fibroblast after treatment with TGF-β . Thus, this investigation sought to determine if TGF-β1 is capable of stimulating activation of fibroblasts as seen in HSc, to examine the potential for auto-induction in these cells and to explore whether IFN was effective in controlling fibroblast activation. Further, the role of IFN-α in antagonizing the autocrine production of TGF-β1 was investigated as one putative mechanism by which IFN counteracts the phenotype of the activated fibroblast observed from HSc.

6.2 MATERIALS AND METHODS

6.2.1 Clinical Specimens

Skin biopsies and serum samples were obtained from patients who had developed HSc characterized by raised, erythematosus, pruritic, thickened, and non-compliant scars confined to the site of thermal injury and had been treated at the Firefighter's Burn Treatment Unit and outpatient burn clinic at the University of Alberta Hospital. After approval by the institutional ethics review board, informed consent was obtained from each patient. Under local anesthesia, biopsies were taken from areas of HSc using a 6-mm punch. A site-matched control biopsy was obtained from uninjured skin in each of the patients. The tissue samples were immediately placed in ice-cold supplemented Dulbecco's modified Eagle medium (DMEM) as described previously (Wang et al, 1996), and transported to the laboratory for processing. Half of each biopsy was used for explant culture and the other half was processed, embedded in paraffin, and sectioned. The serum samples for healthy individuals were obtained from local blood bank and used as normal controls in determination of circulating TGF-β in the patients.

6.2.2 Treatment of HSc Patients with IFN-α2b and Sample Collection

Based on our experience with IFN-γ and -α2b in vitro, we have undertaken a preliminary clinical trial in severely incapacitated burn patients wherein, to date, 3 major burn patients (>25% total body surface area) with significant HSc (>5% total body surface area) have been treated with subcutaneous IFN-α2b (2 x 10^6 Units, 3 x /wk). Punch biopsies of the HSc tissue and blood samples were taken from each patient before, during the IFN treatment at 4 to 5 weeks intervals, and after the treatment. Punch biopsies of the normal skin were taken before and after the treatment. Half of each punch biopsy was frozen in liquid nitrogen, placed on dry ice, and stored at -80°C for RNA extraction. The other half was fixed in 4% paraformaldehyde, embedded in paraffin and sectioned for immunocytochemical studies. The blood samples were kept at 4°C for 1 hr to allow the blood to clot. The serum was separated and stored at -80°C until use.

6.2.3 Cell Culture and Treatment with Cytokines

Dermal fibroblasts were isolated from the punch biopsies by standard explant procedures and cultured in supplemented DMEM as described previously (Tredget et al, 1993). For measurement of TGF-β1 protein, HSc and normal fibroblasts were subcultured in 6-well plates (Corning Laboratory Science Company, Corning, NY) at a density of 150,000 cells per well. After 24 hr, the medium was replaced with fresh medium containing 2% FBS. Sets of triplicate wells in HSc and normal groups were treated with 2000 U/ml of IFN-α2b, IFN-γ 1000 U/ml or a combinations of both for 96 hr. Two other sets of triplicate wells were treated with sterile PBS. The medium in each well was harvested individually for measurement of TGF-β protein and the cells in each were trypsinized and counted with a Coulter Counter (Coulter Electronics, Inc. Hialeah, FL). For determination of the effect of TGF-β1, cells in separate sets of wells were treated with 75 pM recombinant human TGF-β1 for 48 hr. The cells in each well were trypsinized and counted. For isolation of total cellular RNA, HSc and normal fibroblasts were sub-cultured in 100 mm tissue culture dishes (Corning). When the cells reached 70 to 80 per cent confluence, the medium in the cultures was replaced with fresh medium containing 2% FBS. HSc and normal fibroblasts were treated in triplicate with 2000 U/ml of IFN-α2b, 1000 U/ml of IFN-γ, 2000 U/ml of IFN-α2b plus 1000 U/ml of IFN-γ, or sterile PBS as untreated control. The dishes were incubated for an additional 72 hr period under normal culture conditions and the cells were harvested for total cellular RNA.

6.2.4 Determination of Collagen Synthesis in Normal and HSc Fibroblasts

In a separate set of experiments using identical conditions for cytokine treatment, HSc and normal fibroblasts in 6-well tissue culture plates containing 2 ml of medium were exposed to a stable isotope of oxygen, $^{18}O_2$ for 24 hrs, in the presence of TGF-β, IFN-α, or IFN-γ or in combination in a gas-tight chamber. Collagen synthesis, determined by the presence of $^{18}O_2$-labeled hydroxyproline in the conditioned media, was measured by GC/MS as previously described (Tredget et al, 1993). Briefly, the media were extracted with 67% ethanol to separate high-molecular-mass collagenous and other proteins in the precipitate from low-molecular-mass di- and tripeptides or amino acids in the supernatant. The precipitates containing collagen were hydrolyzed and processed for GC/MS (Tredget et al, 1993). The cells were trypsinized and counted with a Coulter Counter (Coulter Electronics, Inc.) and used to determine the rate of collagen synthesis.

6.2.5 Detection of TGF-β in Serum Samples and Cell Culture Media

To determine TGF-β protein in the serum samples and supernatant of fibroblast cultures, we used a sandwich ELISA based on the procedure reported by Danielpour et al (1993). Briefly, 96-well ELISA plates were coated with 100 µl per well of monoclonal antibody against human TGF-β (anti-human TGF-β1, 2, 3; Genzyme, Cambridge MA) at concentration of 1 µg/ml in PBS. The plates were incubated for 2 hr at room temperature followed by 16 hr at 4°C. After washing twice with PBS-Tween-20 (PBS-T), the plates were blocked with 1% bovine serum albumin (BSA, Sigma) for 60 min at room temperature and washed 3 times with PBS-T. The serum samples were extracted using an acid-alcohol extraction procedure (Danielpour et al, 1993). The culture media were acidified with 24 µl per ml of 5N HCl for 15 min at room temperature and neutralized with 40 µl per ml of 1 M HEPES/5 N NaOH (5/2, v/v). One hundred microlitres per well of the extracted serum or acidified/neutralized medium samples were added to the wells of the plates, which were then incubated at room temperature for 60 min. After washing with PBS-T 3 times, the plates were incubated with 100 µl per well of chicken anti-human TGF-β (R & D Systems Inc, Minneapolis, MN) at a concentration of 5 µg/ml for 60 min at room temperature with shaking. After washing 5 times with PBS-T, the plates were incubated with alkaline phosphatase-conjugated rabbit anti-chicken IgG (Sigma) at room temperature for 60 min followed by washing with PBS-T 5 times. After addition of the substrate (o-nitrophenyl phosphate, 1 mg/ml, Sigma), the plates were incubated at room temperature for 60 min and the optical density was read using a THERMO max (Molecular Devices, Menlo Park, CA) microplate reader at wavelength of 405 nm. Serial dilutions (0 to 2500 pg/ml) of recombinant human TGF-β (Genzyme) were used as standards in each plate to quantify TGF-β in the samples.

6.2.6 Isolation of Total RNA from Cells and Tissue

Total cellular RNA was isolated from treated and control cells using the procedure reported by Chomczynski and Sacchi (1987). Briefly, the cells in tissue culture dishes were washed 3 times with ice-cold PBS and lysed with 500 μl of Solution D (Chomczynski and Sacchi, 1987). The lysates were extracted with phenol : chloroform (1:1) followed by chloroform : isoamyl alcohol (49:1). The resulting solutions were then precipitated with ethanol-ammonium acetate and kept at -20°C for 1 hr. After centrifugation, the RNA pellets were re-dissolved in 20 μl of TE buffer (pH 8) and the amounts of RNA were determined by spectrophotometry. Five micrograms of the isolated RNA were run on a 1% denaturing agarose (GIBCO BRL) gel containing 1μg ethidium bromide per ml to confirm that there was no degradation during the isolation procedure.

Total RNA from tissue was isolated using the same procedure with the following modifications: the punch biopsies were weighed, immediately placed into 400 μl of Solution D, minced and homogenized using a Teflon homogenizer. Because the amount of total RNA in small punch biopsies was very low, 20 μg of yeast tRNA was added to each sample as a carrier to allow visualization of the

RNA pellet during processing. Tissue RNA from 5 mg of HSc tissue or 10 mg of normal skin was electrophoresed in an agarose mini-gel and transferred to a nylon membrane, which was then stained with methylene blue (0.03% in 0.3M sodium acetate, pH 5.2). The bands on the stained nylon membrane were scanned and digitized to compare the relative amounts of total RNA from tissues.

6.2.7 Competitive Reverse Transcription-Polymerase Chain Reaction

Specially synthesized competitive complementary RNA (cRNA) templates for human TGF-β1 were employed as internal standards in RT-PCR to quantify the mRNA in HSc and normal fibroblasts and in the tissue after IFN treatment (Chapter Five). First-strand cDNA (RT-cDNA) was made from a fixed amount of total cellular or tissue RNA and varying amounts of internal standard cRNA (IS-cRNA). Six tubes were set up, each containing 1 μg of total cellular RNA or the amount of tissue RNA derived from 200 μg of tissue plus 3.125, 6.25, 12.5, 25, 50, or 100 ng of IS-cRNA. Superscript RNase H^- reverse transcriptase (Superscript RT-I; GIBCO BRL) and sequence specific antisense primer (Chapter Five) for human TGF-β1 were used according to the Superscript RT-I data sheet (GIBCO BRL).

The PCR was carried out in a RoboCycler Gradient 40 temperature cycler (Stratagene Cloning Systems, La Jolla, CA). The gene-specific primers and PCR conditions have been described elsewhere (Chapter Five). The primers were synthesized in the Nucleic Acid Research Center, University of Alberta. The amplified products were analyzed on a 1.2% agarose (GIBCO BRL) gel containing 1 μg/ml ethidium bromide. The sizes of the PCR products were identified by calculating the number of base pairs between the sense and antisense primers complementary to corresponding human TGF-β1 cDNA sequences and indicated by the 100 base pair (bp) DNA molecular weight marker (GIBCO BRL). For positive controls, the plasmid cDNA specific for human TGF-β1 was used as templates in PCR. For negative controls, 200 ng of total cellular RNA plus 20 ng of IS-cRNA without reverse transcription was used.

A non-radioactive quantitative procedure has been used in this experiment to quantify the gene-specific PCR products (Chapter 5). Briefly, the images of the RT-PCR products in agarose gels were scanned with an Apple One Scanner connected to a StarMax 4000/160 computer and digitized with the NIH Image v1.6.1 program. The intensities of the bands for the internal standards and TGF-β1 mRNA were plotted against the amount of IS-cDNA in PCR reactions and the amount of RT-cDNA for TGF-β1 was calculated. The amount of TGF-β1 mRNA per μg of total cellular RNA was calculated based on the amount of internal standard in each PCR reaction and corrected with the molecular weight of internal standards and the TGF-β1 PCR products. The number of copies of TGF-β1 mRNA per cell was calculated assuming that a nucleated mammalian cell expresses 10 pg of total RNA (Kanangat et al, 1992).

6.3 RESULTS

6.3.1 The Effect of TGF-β1 and IFNs on Proliferation of Dermal Fibroblasts

Treatment of non-confluent normal and HSc fibroblasts with IFN-α or -γ in the absence of TGF-β1 inhibited cell proliferation. A weak additive effect was observed when both IFN-α and -γ were used in HSc cells ($p < 0.05$, $n = 6$, paired t test) but not in normal fibroblasts (**Figure VI-1A**). However, they had no effect on near confluent cells (data not shown). Treatment of HSc and normal fibroblasts with recombinant human TGF-β1 stimulated cellular proliferation in a dose-dependent fashion (Data not shown). TGF-β1 not only stimulated the non-confluent (at day 4 in culture) fibroblasts to proliferate, but also stimulated the proliferation of near-confluent cells (at day 12 in culture) in vitro (data not shown). Either IFN-α2b or IFN-γ antagonized the stimulatory effects of TGF-β1 on fibroblast proliferation. Treatment of HSc and normal fibroblasts with both IFNs in the presence of TGF-β1 had a weak additive effect on the TGF-β-induced cell proliferation (**Figure VI-1B**).

6.3.2 The Effect of TGF-β and IFNs on Collagen Synthesis by Dermal Fibroblasts

Collagen synthesis, as determined by analysis of ^{18}O-labelled hydroxyproline in the medium, was greater in HSc fibroblasts than in their normal counterparts. TGF-β1 stimulated collagen production by both HSc and normal fibroblasts in a dose-dependent manner with the maximal effect at 75 pM (**Figure VI-2A**). On the other hand, IFN-α2b and -γ both inhibited collagen production and together had a weak additive effect (**Figure VI-2B**). Treatment of both HSc and normal fibroblasts with IFNs inhibited the TGF-β stimulated collagen production (**Figure VI-2C**).

6.3.3 The Effect of IFNs on TGF-β Protein Production by Hypertrophic Scar and Normal Fibroblasts

Using a sandwich ELISA procedure, we compared the TGF-β protein secretion by HSc and normal fibroblasts in vitro. Five pairs of HSc and normal fibroblasts were examined. All 5 strains of HSc cells produced significantly more TGF-β than their counterpart normal cells. The secretion of TGF-β was significantly reduced in the media after IFN treatment (**Figure VI-3**). Both IFN-α2b and -γ were effective in inhibiting the amount of TGF-β1 secreted by both HSc and normal fibroblasts. A combination of IFN-α2b and -γ had a weak additive effect (**Figure VI-3**).

6.3.4 The Effect of IFN-α2b on the Circulating TGF-β Levels in HSc Patients

A total of 26 serum samples from healthy individuals were examined for the presence of TGF-β using the sandwich ELISA for human TGF-β1, 2, 3 (**Figure VI-4A**). The mean value of these samples plus/minus 2 standard deviations (± 2 SD) was considered as the normal range for TGF-β in the circulation. A pooled sample of these sera was used as a reference in later experiments for determination of TGF-β in the serum of patients. The peripheral blood of HSc patients contained significantly higher levels of TGF-β than healthy individuals. A total of 12 patients were examined. Most of them had levels of TGF-β in their serum, which were outside the normal range before IFN treatment. The serum TGF-β levels of three patients whose tissue samples were examined for TGF-β mRNA were shown in **Figure VI-4A**. Treatment with IFN-α2b significantly reduced the serum TGF-β levels to within the normal range during and shortly after treatment (**Figure VI-4A**). **Figure VI-4B** represents one typical patient whose serum TGF-β was measured at monthly intervals before, during and after IFN-α2b treatment.

6.3.5 Fibroblasts Possess TGF-β mRNA and its Expression is Inhibited by IFN-α and -γ

Using quantitative RT-PCR (described in Chapter Five), the number of copies of TGF-β1 mRNA per cell was determined. This was significantly higher in the HSc cells than in their counterpart normal dermal fibroblasts (Data not shown). Both IFN-α2b and -γ reduced the mRNA but the greatest reductions were seen when both were used (**Figure VI-5A, -5B, -5C, and -5D**). All 3 pairs of HSc and normal fibroblasts behaved in a very similar fashion (**Figure VI-5E**). Overall, HSc fibroblasts possess significantly more TGF-β1 mRNA than their counterpart normal cells, and IFN treatment significantly reduced the mRNA expression by both HSc and normal cells.

6.3.6 The Effect of IFN-α2b on TGF-β mRNA in HSc Tissue

HSc tissue contained significantly more mRNA for TGF-β1 than did normal skin, as determined by the competitive RT-PCR (**Figure VI-6A**). A total of 3 pairs of punch biopsies from 3 patients was examined and similar results were obtained (**Figure VI-6A**). Treatment of the patients with IFN-α2b decreased the TGF-β1 mRNA in the tissue within 1 month. Examination of serial samples from the patients during treatment revealed that TGF-β mRNA decreased even after the first injection and remained at a level very close to the normal throughout treatment (**Figure VI-6A**). **Figure VI-6B** shows TGF-β1 mRNA in the HSc and normal tissues of a representitive patient before, during and after IFN-α2b treatment.

6.4 DISCUSSION

Although many factors have been implicated in the formation of post-burn HSc, its pathogenesis is still unclear. It is well-known that cytokines play a critical role in the regulation of cellular activities and ECM metabolism. Among the fibrogenic cytokines, TGF-β1 is important in that it has profound effects on fibroblast proliferation and collagen synthesis. On the other hand, IFNs represent a group of anti-fibroproliferative agents that inhibit fibroblast proliferation and collagen production. These two groups of cytokines appear to work in opposite directions in regulating ECM metabolism. Demonstration and confirmation of these kinds of antagonism of cytokine effects in wound healing could provide a theoretical basis for designing preventive and therapeutic protocols for HSc.

We have previously demonstrated that fibroblasts derived from HSc tissues produce more mRNA and protein for TGF-β, type I and type III procollagens than their normal counterpart (Ghahary et al, 1993; 1994). It is possible that higher levels of TGF-β induce the over-production of collagens associated with HSc formation. This assumption is supported by the findings that fibroblasts produce TGF-β and possess cell surface receptors for TGF-β (Falanga et al, 1994), and that the promoter regions of the type I and type III collagen genes contain TGF-β-responsive elements (Chung et al, 1996; Rossi et al, 1988). TGF-β produced by fibroblasts could have an autocrine effect, as has been demonstrated in murine mesangial cells (Ziyadeh, et al, 1994). Agents blocking this autocrine loop would reduce collagen synthesis and hopefully have therapeutic effects in HSc. Neutralizing antibodies against TGF-β reduce collagen expression by fibroblasts in vitro (Sharma et al, 1996) and decorin, a natural inhibitor of TGF-β, decreases scarring in experimental kidney disease (Border et al, 1992). However, neutralizing antibodies cannot be used in vivo for treatment of fibroproliferative disorders due to the antigenicity of these proteins. Thus, it is necessary to look for other types of anti-fibrogenic agents.

Interferons appear to be good candidates for this purpose. We and others have previously demonstrated that type I and type II IFNs inhibit proliferation and collagen synthesis in fibroblasts (Duncan et al, 1995; Harrop et al, 1995). They have also been shown to enhance the synthesis of the metalloproteinases collagenase and stromelysin by fibroblasts (Duncan and Berman, 1989; Tamai et al, 1995). It has been speculated that the anti-fibrogenic effects of IFNs are mediated by inhibiting collagen synthesis (Duncan et al, 1995; Harrop et al, 1995), enhancing metalloproteinase production (Duncan and Berman, 1989; Tamai et al, 1995) and antagonizing the fibrogenic effects of TGF-β (Gurujeyalakshmi and Giri, 1995). In this study we demonstrated that either IFN-α2b or -γ alone were effective in inhibition of cell proliferation and collagen synthesis. The apparent additivity of the effects of IFN-α2b and -γ suggests that these two types of IFNs could be used in combination to achieve maximal efficacy. It is of interest that IFNs can antagonize TGF-β-induced cell proliferation and collagen synthesis. This antagonism was observed with IFN-α2b and -γ alone or in combination, indicating that type I and type II IFNs use a common mechanism to antagonize the effects of TGF-β although they use distinct pathways for signal transduction (Larner and Reich, 1996; Schindler et al, 1992). Binding of IFNs to cell-surface receptors triggers a cascade of kinase-mediated signaling leading to the activation of certain genes, resulting in growth arrest of the cells and shutdown of the synthesis of certain proteins, including collagen.

Autoinduction has been observed in TGF-β-mediated signaling. This was demonstrated by enhanced TGF-β gene expression and protein synthesis in the presence of TGF-β in culture. It is thought to be important for amplification of TGF-β initiated signals (Kim et al, 1990). We speculate that, besides their direct inhibitory effect, IFNs affect the autoinduction of TGF-β, resulting in lower TGF-β gene expression and protein production by fibroblasts. This lower endogenous TGF-β may lead to lower proliferation and reduced collagen production by the cells. This speculation is supported by the evidence that the TGF-β1 gene promoter region contains sequences homologous to IFN-responsive elements (Kim et al, 1989b) and that IFNs inhibit TGF-β mRNA expression and protein production in vitro. The finding that treatment of HSc patients with IFN-α2b appears to reduce TGF-β mRNA in the tissue and protein in the circulation may also support this assumption.

Quantification of gene expression in small punch biopsies is now possible due to development of quantitative RT-PCR techniques. We have developed a simple yet reliable procedure to construct gene-specific cRNA templates to be used as internal standards in RT-PCR to quantify mRNAs (Chapter Five). This procedure has several advantages for quantitative RT-PCR. Firstly, the synthetic cRNA template is gene-specific and only 63 bases longer than the mRNA. It uses the same primer-binding sequences as TGF-β1 mRNA from cells or tissue. This should eliminate variations in efficiencies of primer binding and amplification. Secondly, the internal standard cRNA template is added before reverse transcription reaction. This should eliminate differences in efficiency of reverse transcription. Thirdly, the non-radioactive nature of this procedure makes it safer to use. The results obtained by quantitative RT-PCR are comparable to those obtained by Northern analysis and ELISA, indicating that this procedure is a viable method for quantification of gene expression in a small amount of tissue.

In conclusion, IFN-α2b and -γ inhibit proliferation and collagen synthesis of human dermal fibroblasts, while TGF-β had stimulatory effects which were antagonized by IFN-α2b or IFN-γ. Additive effects were observed with both IFN-α2b and -γ in inhibition of cell proliferation and collagen synthesis, and in antagonizing the stimulatory effects of TGF-β. Subcutaneous injection of HSc patients with IFN-α2b reduced TGF-β mRNA levels in the HSc tissue and decreased TGF-β protein in the serum.

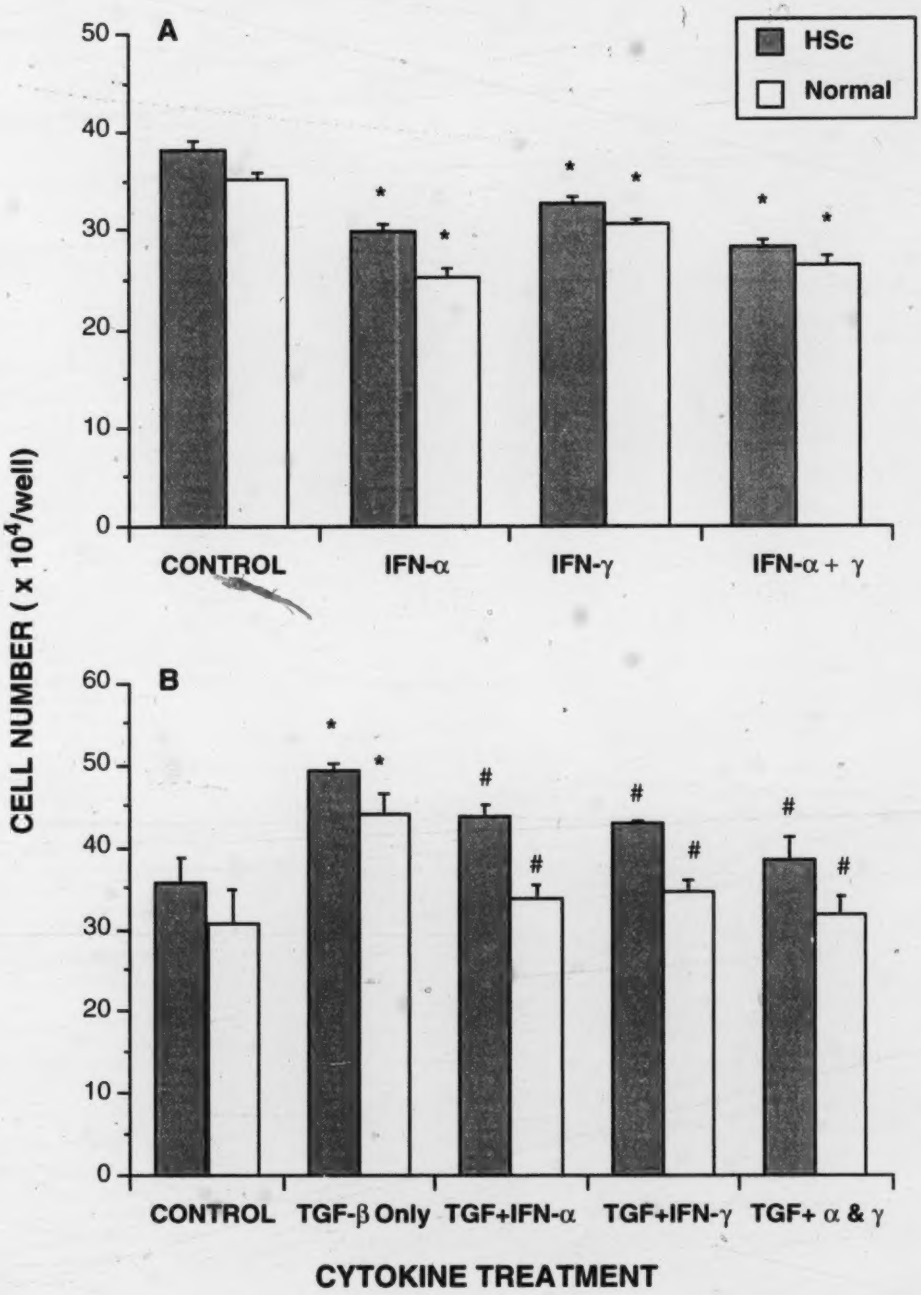


Figure VI-1. See next page for legend.

Figure VI-1. **The Effect of TGF-β and IFNs on Cell Proliferation of HSc and Normal Fibroblasts**. Sub-confluent HSc and normal fibroblast cultures were treated with 2000 units per ml of IFN-α2b, or 1000 units per ml of IFN-γ or both in the presence or absence of 75 pM of TGF-β for 24 or 96 hrs in DMEM with 2% FBS as described in "Materials and Methods". The cells were trypsinized and counted. Panel A shows the cell proliferation after IFN treatment in the absence of TGF-β. Asterisks indicate significant difference when compared to respective control group ($p < 0.05$, $n = 6$, paired t test). Panel B shows the cell proliferation after treatment with IFNs in the presence of TGF-β. Asterisks indicate significant difference between the IFN-treated groups and the control group in Panel A and between TGF-β-treated groups and the control group in Panel B. The symbol # indicates significant difference between the IFN-treated groups in the presence of TGF-β and TGF-β alone groups ($p < 0.05$, ANOVA). Bars indicate standard error of the mean (SEM).

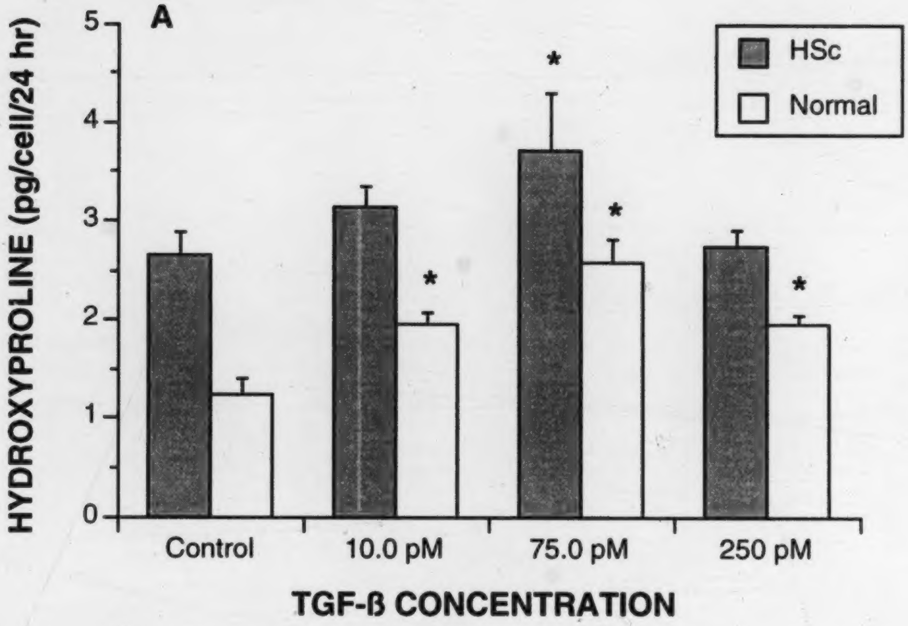
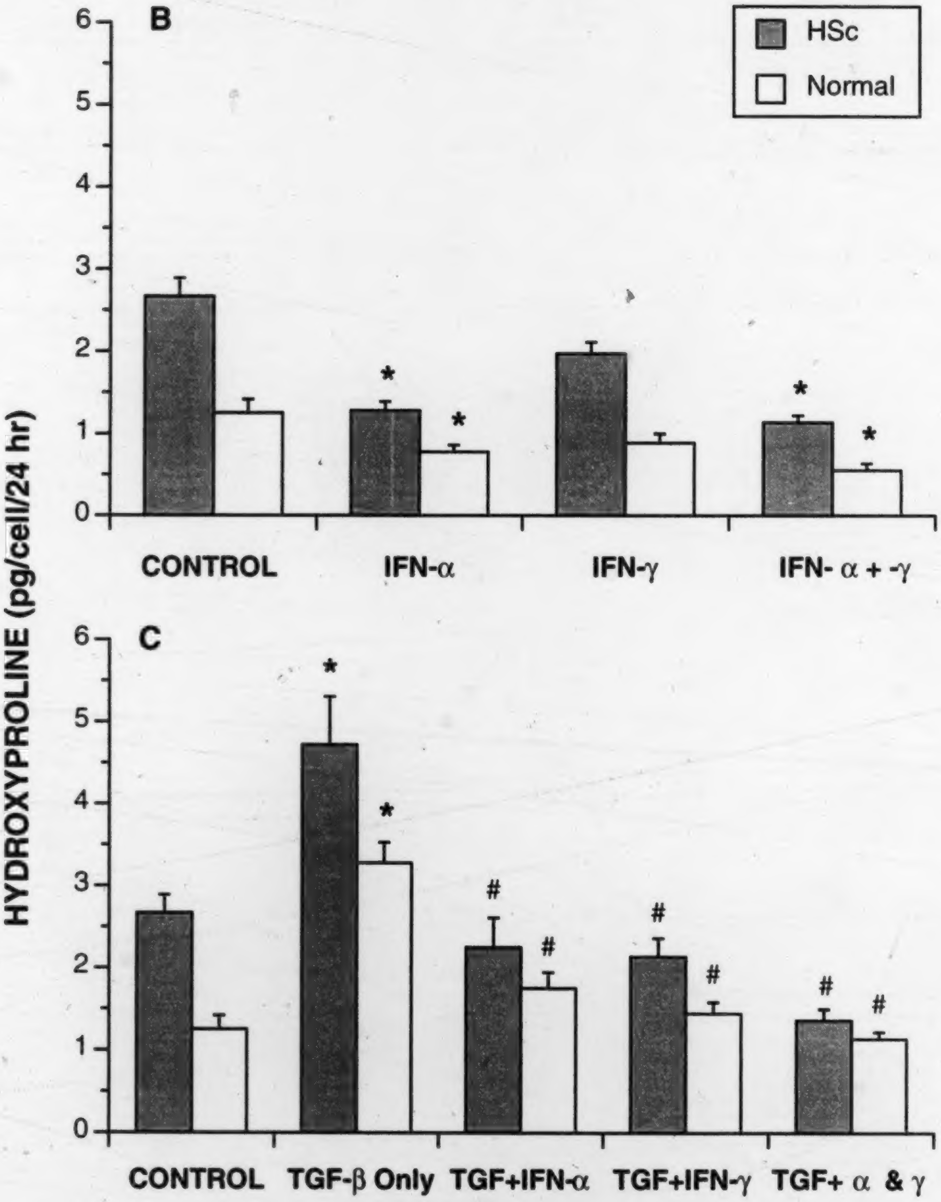


Figure VI-2. **The Effect of TGF-β and IFNs on Collagen Synthesis of HSc and Normal Fibroblasts**. Near confluent HSc and normal fibroblasts were treated with TGF-β for 24 hr, and with IFN-α2b or -γ or both in the presence or absence of TGF-β in 6-well plates for 72 hr. The cells were than exposed to $^{18}O_2$ in a gas-tight chamber for 24 hr. The medium was harvested and $^{18}O_2$ incorporated into hydroxyproline was quantified by GC/MS. Panel A shows the dose response of HSc and normal cells to TGF-β1 treatment. Panel B shows the hydroxproline synthesis of the cells after IFN treatment in the absence of TGF-β. Panel C shows the hydroxproline synthesis of the cells after IFN treatment in the presence of TGF-β. Bars indicate the SEM. Asterisks indicate significant difference between IFN-treated groups and the control groups. The symbol # indicates significant difference between IFN plus TGF-β-treated groups and TGF-β only groups ($p < 0.05$, ANOVA). Panels B and C are on next page.


B
HSc
Normal
6
5
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3
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IFN-α
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CONTROL
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TGF+IFN-α
TGF+IFN-γ
TGF+ α & γ
HYDROXYPROLINE (pg/cell/24 hr)
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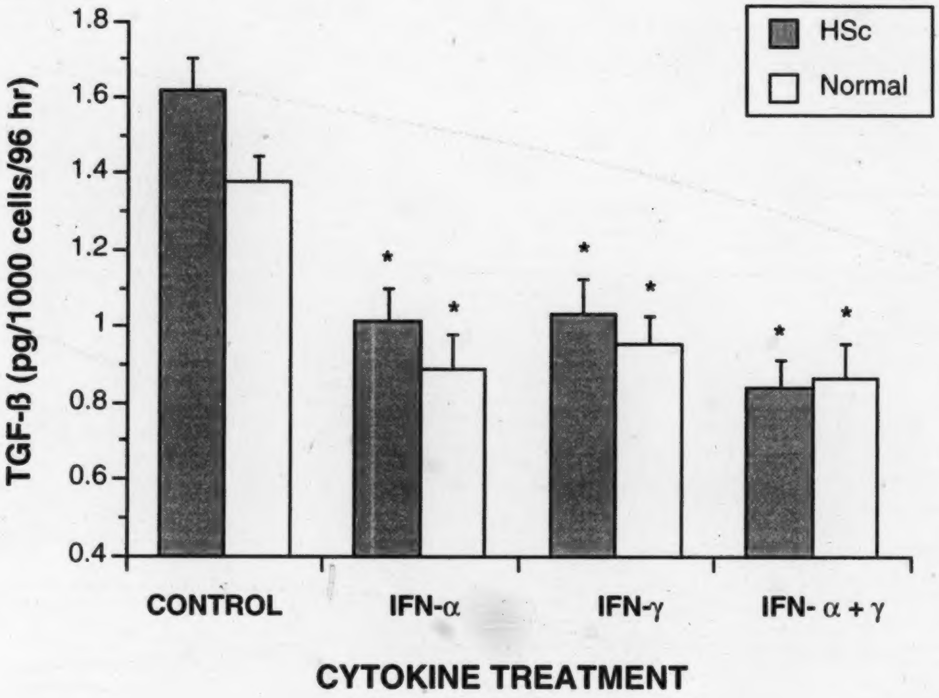

Figure VI-3. **Sandwich ELISA for TGF-β Secretion by HSc and Normal Fibroblasts after IFN Treatment.** Near confluent HSc and normal fibroblasts were treated with IFN-α2b, -γ or both in DMEM containing 2% FBS for 96 hr. TGF-β in the medium was measured with a sandwich ELISA. The amount of TGF-β was calculated and expressed as pg of TGF-β protein per 1000 cells per 96 hr. The amount of TGF in 2% FBS was subtracted from the total amount of TGF-β in the medium. Bars indicate the SEM. Asterisks indicate significant difference when compared with the control groups ($p < 0.05$, $n = 5$, paired t test).

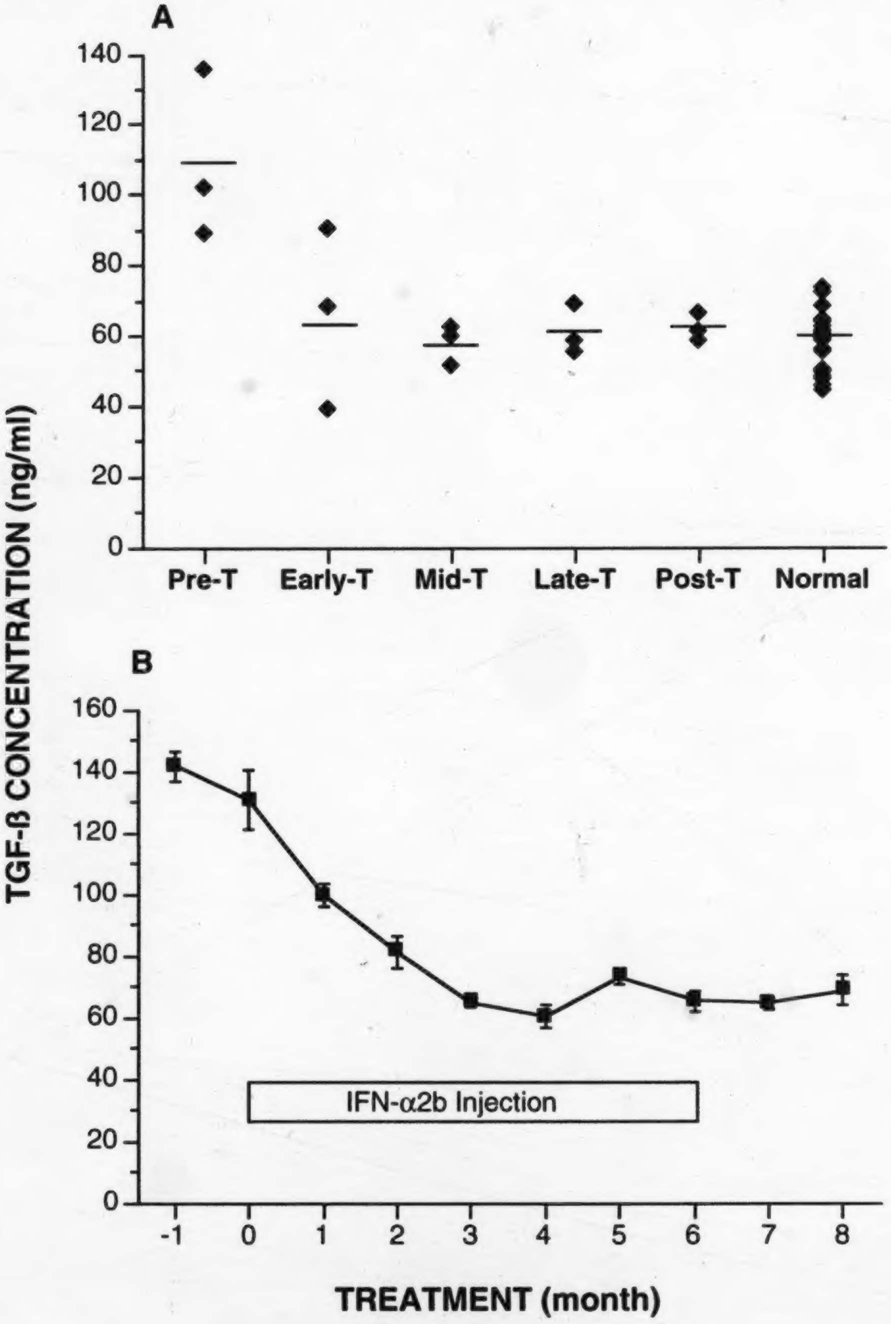

Figure VI-4. See legend on next page.

Figure VI-4. **Sandwich ELISA for TGF-β in the Serum of Normal and HSc Patients after IFN Treatment.** The sera from normal individuals and HSc patients were extracted with the acid-alcohol procedure and assayed for TGF-β using a sandwich ELISA. Panel A shows TGF-β levels in the sera of 3 IFN-α2b-treated patients. The data are presented as pre-treatment (Pre-T), treatment at the early stage (Early-T, 1 to 2 months), middle stage (Mid-T, 3 to 4 months), late stage (Late-T, 5 to 6 months), and post-treatment (Post-T). The normal group shows the TGF-β levels in the sera of 26 healthy individuals. Panel B shows the kinetics of TGF-β levels in one of the IFN-α2b treated patients before, during and after IFN-α2b treatment. Bars indicate the SEM of triplicate experiment.

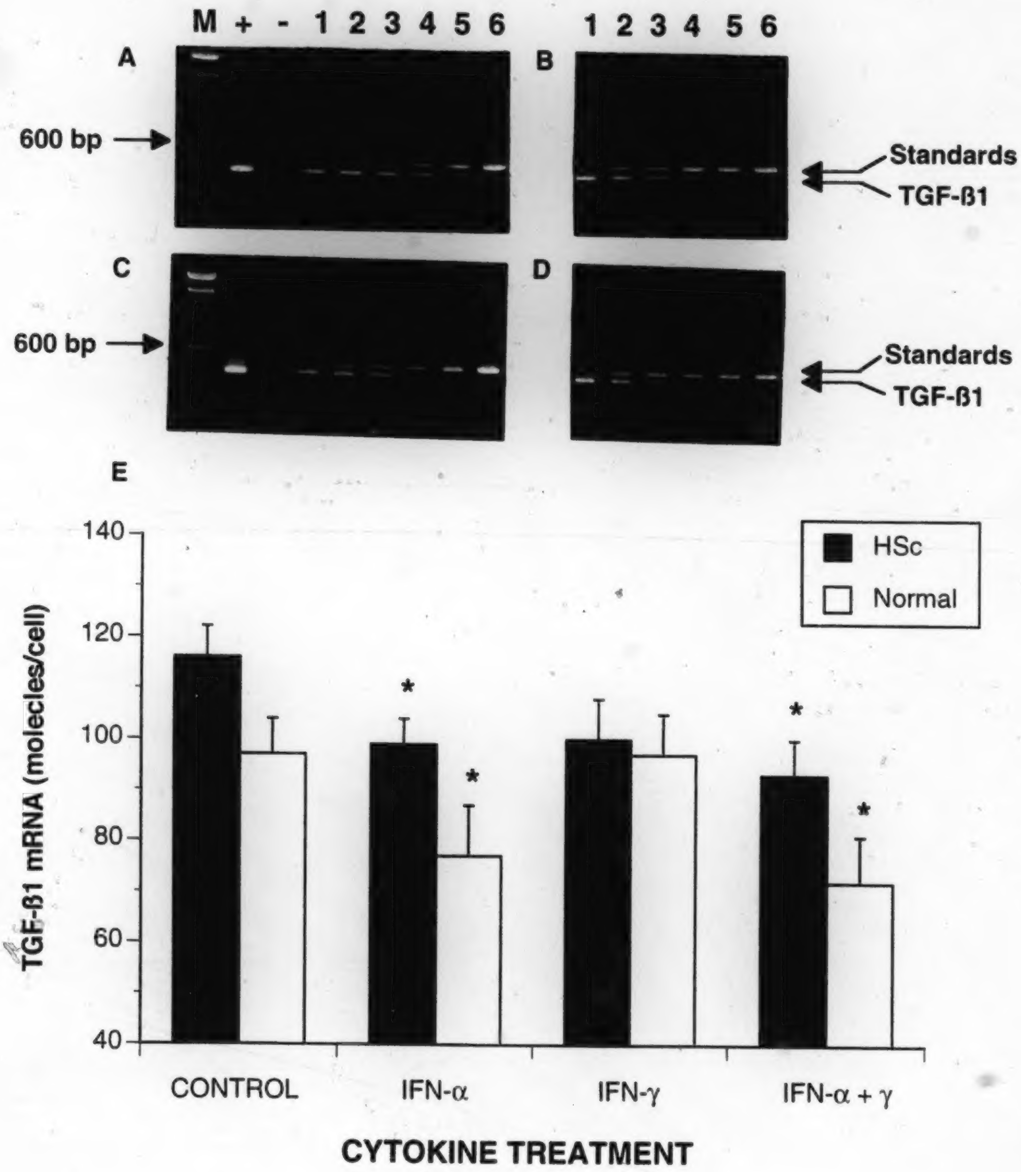

Figure VI-5. See legend on next page.

Figure VI-5. **Quantitative RT-PCR for TGF-β1 mRNA in HSc and Normal Fibroblasts after IFN Treatment.** Near confluent HSc and normal fibroblasts were treated with IFN-α2b, -γ or both for 72 hr in DMEM with 2% FBS. Total RNA was extracted and TGF-β1 mRNA was determined with the quantitative RT-PCR using TGF-β1 gene specific cRNA as a standard. Panels A to D show the TGF-β1 RT-PCR products in ethidium bromide-stained agarose gel. Lanes M in panels A and C carry the 100 bp DNA size marker. Lanes labelled " + " are the positive controls using plasmid cDNA containing human TGF-β1 sequence as templates in PCR. Lanes labelled "-" are the negative controls using total cellular RNA and internal standard cRNA without reverse transcription as templates in PCR. Lanes 1 to 6 in Panel A are the RT-PCR bands for the control group using different amount of internal standard cRNA. Lanes 1 to 6 in Panel B are the bands for the IFN-α2b treated group. Lanes 1 to 6 in Panel C are the bands for the IFN-γ treated group. Lanes 1 to 6 in Panel D are the bands for the IFN-α2b plus -γ treated group. Panel E shows the quantitative data derived from 5 pairs of HSc and normal cells. Asterisks indicate significant differences between control and treated groups ($p < 0.05$; ANOVA). Bars indicate the SEM of the 5 strains HSc and normal cells.

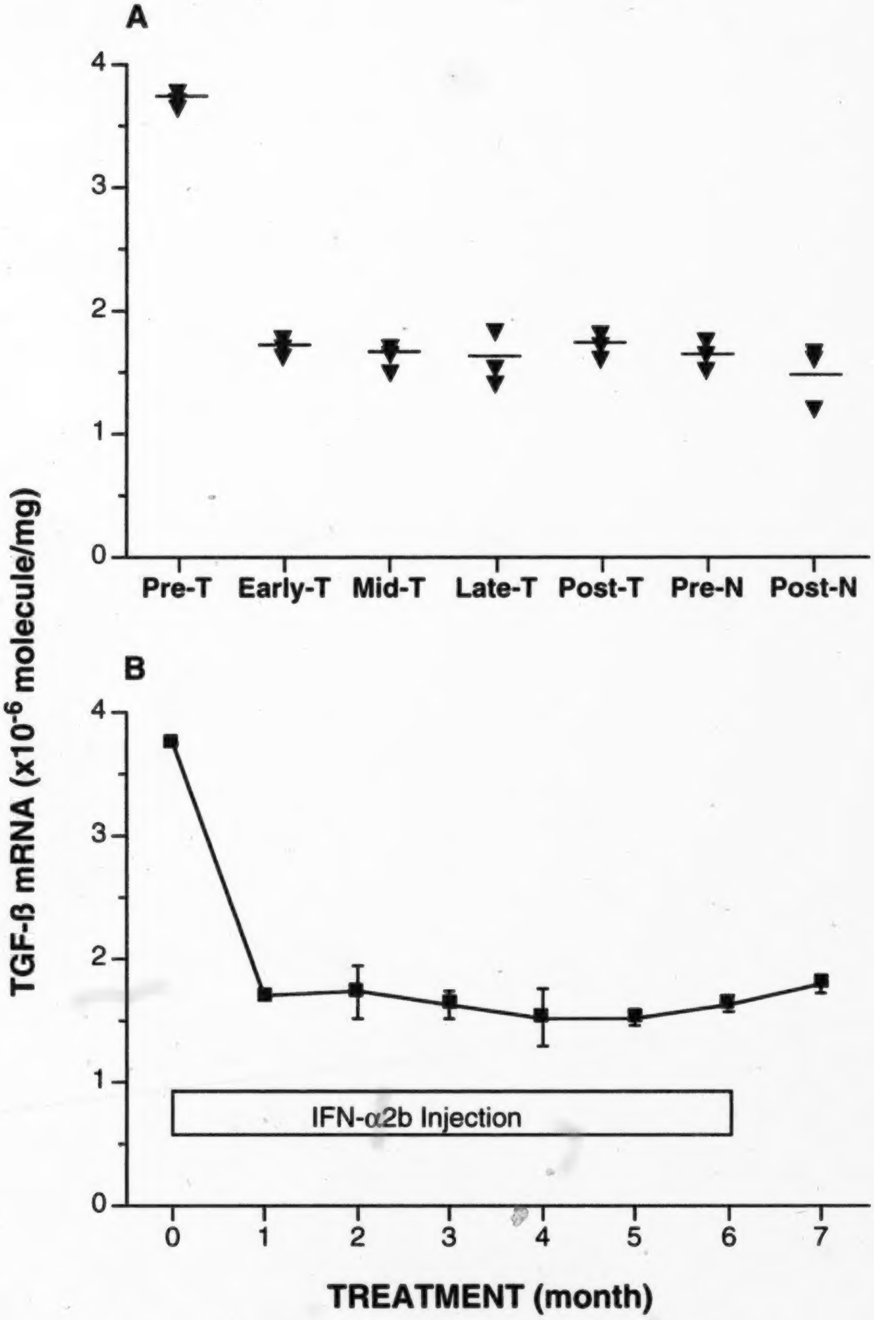

Figure VI-6. See legend on next page.

Figure VI-6. **Quantitative RT-PCR for TGF-β mRNA in HSc and Normal Tissue after IFN Treatment.** Total RNA was extracted from serial punch biopsies of scars and normal skin from IFN-α2b treated HSc patients. TGF-β1 mRNA in the samples was determined with the quantitative RT-PCR procedure as described in "Materials and Methods". Panel A shows the data from 3 sets of punch biopsies derived from IFN-α2b treated patients. TGF-β1 mRNA was expressed as number of molecules per mg of tissue (wet weight). The data were presented as pre-treatment group (Pre-T), treatment at early stage (Early-T, 1 to 2 months), middle stage (Mid-T, 3 to 4 months), late stage (Late-T, 5 to 6 months), and post-treatment (Post-T). The normal controls were punch biopsies from the normal skin of the patients pre- (Pre-N) and post-treatment (Post-N). Panel B shows the kinetics of TGF-β1 mRNA in the HSc tissues of a representative set of punch biopsies from one IFN-α2b treated patient. Bars indicate the SEM of triplicate experiment.

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CHAPTER SEVEN

GENERAL DISCUSSION AND CONCLUSION

7.1 DISCUSSION

Disruption of the integrity of the dermis triggers a series of well-regulated events leading to healing of the wound and formation of a scar. Both cellular and ECM components of the skin are involved in these events. Among the participating cells, fibroblasts play a central role in wound healing and scar formation since they produce both structural and regulatory proteins and other molecules that are essential for the reconstitution of the damaged tissues. Many other factors also actively participate in and regulate these events to ensure maximal healing with minimal scar formation. Accumulating evidence indicates that NO, type beta TGFs and IFNs are three regulatory factors involved in wound healing and scar formation. It has been demonstrated that there are synergistic or antagonistic interactions between these factors. Mutual regulation between them has also been observed. The present study was conceived to gather more information about the regulation of wound healing and mechanisms of HSc formation.

In Chapter Three I demonstrated that human dermal fibroblasts have the capability of expressing both eNOS and iNOS and producing NO *in vitro*. Although rat and mouse fibroblasts had been demonstrated to express NOS and produce NO, this is the first report that human fibroblasts have the capability of doing so. Demonstration of eNOS expression by fibroblasts raises the possibility that these matrix protein producing cells are also important in regulating the blood circulation in the scar tissue and the cellularity of the scar because NO is well-known to have vasodilatory and anti-proliferative effects. These findings established the foundation for the assumption that fibroblasts are actively involved in the inflammatory response during wound healing since NO is well-known to be produced during inflammatory responses. At the same time, these findings also support the postulate that NO is involved in wound healing in that fibroblasts are the most abundant cell population during wound repair.

One of the major roles of NO in wound healing may be to accelerate the wound healing process. This may be achieved by stimulating collagen synthesis and deposition in the wound (Kirk et al, 1993) and by increasing the blood supply of the wound through its vasodilatory effect (Altman et al, 1994). In fact, the involvement of arginine, the substrate for NO biosynthesis, in wound healing was demonstrated in the late 70's, about 10 years before NO was recognized as a bioactive molecule. In 1978, Seifter and associates reported that depletion of arginine in the diet retarded wound healing while supplementation with arginine accelerated this process. They concluded that arginine is an essential amino acid in the injured animal (Seifter et al, 1978). Later, they found that arginine stimulated wound healing in humans (Barbul et al, 1983). However, the mechanism of action of arginine was not clear until the arginine-NOS pathway was discovered in the late 80's and the findings that NO stimulated collagen synthesis and deposition (Kirk et al, 1993). It is now clear that NO, the metabolite of arginine in this pathway, plays a role in wound healing.

It is intriguing that NO and its derivative peroxynitrite also activates the metalloproteinases collagenase and stromelysin (Murrell et al, 1995; Trachtman et al, 1996). This finding raises the possibility that NO is also involved in tissue remodeling and HSc formation. On one hand, NO enhances collagen synthesis and deposition and increases blood flow of the wound, resulting in accelerated healing. On the other hand, it increases metalloproteinase production and activation leading to enhanced proteolysis of ECM. These increases in both collagen synthesis and collagen degradation result in an enhanced collagen turnover, which is critical for scar remodeling. Thus, it can be speculated that deficiency in NO may affect the remodeling process of wound healing, contributing to HSc formation. The findings described in Chapter 4 support this assumption in that HSc fibroblasts express less eNOS and produce less NO than those from normal dermis. The deficiency in NO synthesis by fibroblasts may lead to lower levels of NO in the tissue, resulting in impaired collagen turnover and excessive proliferation of fibroblasts since NO normally inhibits cell proliferation (Goureau et al, 1993). It is assumed that NO produced by fibroblasts may be more important in regulating collagen turnover and fibroblast proliferation than that produced by other cells because endogenously synthesized NO may directly act on gene expression and cell cycle control. Due to its short half-life and rapid diffusion, local or endogenous production is very important for NO to achieve its biological activities.

The deficiency in NO production in HSc fibroblasts may be due to a phenotypic alteration of the fibroblast. This assumption is based on the findings that phenotypic alterations have been observed in human dermal fibroblasts in aspects of collagen synthesis and collagenase production (Ghahary et al, 1993; 1996). The phenotypic alteration of the cells may be a result of altered cytokine profile in HSc tissues and subsequently in cell cultures. This speculation is based on the fact that most cellular activities, including NO production, are governed by a network of cytokines. These act synergistically or antagonistically on certain activities of the cells to maintain appropriate function. At the same time, cytokine production is itself controlled by a network of regulatory pathways including autocrine, paracrine, endocrine, and feed-back mechanisms. In the case of NO production, both stimulatory and inhibitory regulation has been observed. Proinflammatory cytokines such as IFNs, IL-1, TNFs and bacterial LPS stimulate production of NO via iNOS while type beta TGFs, IL-4 and IL-10 are inhibitory. These factors work together to control NO production by the cells.

It is hypothesized that the lower levels of NO production by HSc cells could be due to higher levels of TGF-β expression by the same cells and that IFNs, which stimulate NO production, may antagonize the effect of TGF-β. This speculation is based on the findings that HSc fibroblasts express more mRNAs for TGF-β1, type I and III pro-collagens (Ghahary et al, 1993). To test these hypotheses, a series of experiments was designed. The results described in Chapter 5 support these hypotheses in that fibroblasts derived from HSc tissue not only express more mRNA for TGF-β1 as demonstrated before (Ghahary et al, 1993), but also produce more TGF-β protein *in vitro* as determined by ELISA. Furthermore, higher levels of TGF-β1 mRNA were detected in HSc tissue in comparison with normal skin, indicating that TGF-β is more abundant in HSc tissue *in vivo*. This reverse-correlation between NO production and TGF-β synthesis suggests that TGF-β may be important in regulating local NO production in HSc tissues *in vivo*. It has been well-documented that TGF-β inhibits NO production by endothelia, epithelia, and macrophages (Goureau et al, 1993; Inoue et al, 1995; Vodovotz et al, 1993). These observations support the speculation that TGF-β plays a negative-regulatory role in NO production by fibroblasts. However, a direct effect of TGF-β on NO production by human fibroblasts has not yet been demonstrated.

A new quantitative RT-PCR procedure has been developed in this study. This procedure is based on the theory that DNA templates with the same primer binding sequences compete with each other for primer binding, DNA polymerase and nucleotides in the PCR reaction. This procedure requires an internal standard DNA that has the same primer binding sequences as the target molecule but a different length of DNA between them. Serial dilutions of a known amount of internal standard DNA were co-amplified with a fixed amount of target molecule to generate a graded series of bands for internal standard and a reversed series for the target molecule, due to competition. The equivalence point of these two series of bands was used to calculate the amount of target molecule in the reaction. The number of target molecules per cell was calculated on the basis that each nucleated mammalian cell produces about 10 pg of total RNA (Kanangat et al, 1992). This procedure started with a gene-specific PCR product, in which a unique restriction site was identified and used to insert a small cDNA fragment to make it longer than the original molecule. The modified cDNA was amplified by PCR and subcloned into a plasmid containing T7 and T3 bacterophage RNA polymerase promoter sequences. To eliminate the difference due to efficiency of reverse transcription, the gene specific cRNA was synthesized and used in reverse transcription reactions. There are several distinct advantages to this procedure. Firstly, the primer binding sequences for the internal standard are exactly the same as those of the target molecule. This should ensure the competition between the internal standard and target molecule in the PCR reaction. Secondly, the RT-PCR product for the internal standard is only 50 to

100 bp different from that for the target molecule. This should minimize differences due to efficiency of PCR reaction. Thirdly, this procedure does not use radioactive materials, thus it is safer to use and more environmentally sound. Finally, it only requires a single subcloning step. These advantages make this procedure a convenient and reliable method for quantification of gene expression in small number of cells or small amounts of tissue.

To validate this procedure, I compared TGF-β mRNA expression in HSc fibroblasts with that in normal cells. The results showed that the fibroblasts derived from HSc tissue contained significantly higher numbers of TGF-β mRNA molecules than normal cells. This was confirmed by Northern analysis, the well-established method for quantification of gene expression. The data derived from ELISA for TGF-β protein also showed similar alteration between HSc and normal cells. These results indicate that the newly developed competitive RT-PCR is a valid method for quantification of gene expression. Using this method, I examined TGF-β mRNA in HSc tissue in comparison to that in normal skin. The data demonstrated that more TGF-β mRNA was present in HSc tissue, indicating that, like HSc cells, HSc tissue also possesses more TGF-β transcripts than normal skin.

It has been demonstrated that both type I and type II IFNs inhibit the proliferation and ECM production by mesenchymal cells *in vitro*. The mechanisms of anti-proliferative effect of IFN are quite clear. Upon binding to receptors on the cell surface, IFNs trigger a group of tyrosine kinases in the cytoplasmic domains of the receptors, leading to phosphorylation of an array of functional proteins. The IFN-activated nuclear factors regulate the expression of cell cycle control genes such as cyclin E and cdk inhibitors, a group of proteins/protein kinases responsible for cell cycle control, resulting in arrest of the cells in G1 (Hobeika et al, 1997; Zhang and Kumar, 1994). On the other hand, the mechanisms of the anti-fibrogenic effects of IFNs are not clear, although they have been reported to down-regulate the mRNAs for procollagens and up-regulate the mRNA for collagenase (Ghahary et al, 1993). It is assumed that alpha IFNs may exert their effects indirectly. This assumption is based on the observation that IFN-α2b require a minimum of 72 hr to achieve its maximum inhibitory effect on procollagen mRNA expression (Ghahary et al, 1993). One of the possible indirect mechanisms of IFNs may be through inhibition of TGF-β production and autocrine action. The data in Chapter 6 supported this assumption in that IFN-α2b treatment down-regulated TGF-β mRNA and protein production by both HSc and normal fibroblasts *in vitro*.

It would be of interest to know if IFNs have the capability of inhibiting TGF-β mRNA and protein production *in vivo*. The data on tissue obtained from IFN-α2b treated patients showed that both mRNA and protein were decreased after IFN-α2b treatment, as determined by quantitative RT-PCR for TGF-β mRNA in punch biopsies and ELISA for TGF-β proteins in the serum. These results suggest that IFN-α2b inhibits TGF-β production by dermal fibroblasts both *in vitro* and *in vivo*. These findings also support the assumption that IFNs have an indirect effect on ECM metabolism through their inhibitory action on TGF-β production.

7.2 CONCLUSIONS

This thesis presents data that provide information about the physiology and gene expression of human dermal fibroblasts and their responses to IFN-α2b, -γ and

TGF-β treatment. This study also made a considerable contribution to our understanding of the roles of NO in wound healing and HSc formation and the possible mechanisms of the anti-fibrogenic effect of IFN. The following is a list of the significant findings:

I. Human dermal fibroblasts spontaneously produce NO *in vitro* and the NO production can be increased by IFN-γ plus LPS treatment.

II. The fibroblasts use the arginine-NOS pathway to synthesize NO.

III. The data from the citrulline assay, RT-PCR and immunocytochemistry show that these cells express both eNOS and iNOS.

IV. Fibroblasts derived from HSc tissues produce less NO than cells derived from normal skin.

V. HSc fibroblasts express less eNOS than normal cells and iNOS expression is not altered.

VI. A simple and reliable procedure has been developed to construct gene specific internal standard for quantitative RT-PCR.

VII. HSc tissues and fibroblasts produce more mRNA for TGF-β1 than normal skin and cells as determined by the quantitative RT-PCR method.

VIII. HSc fibroblasts synthesize more TGF-β protein than normal cells *in vitro* as determined by ELISA.

IX. IFN-α2b and -γ inhibit TGF-β mRNA and protein production by both HSc and normal fibroblasts *in vitro*.

X. IFN-α2b treatment reduces TGF-β1 mRNA in HSc tissue as determined by quantitative RT-PCR and TGF-β protein in serum as determined by ELISA.

7.3 FUTURE PERSPECTIVES

The role of NO in wound healing is a new area of research receiving more and more attention. It has been reported that NO accelerates wound healing and increases collagen synthesis and deposition in the wound. However, the research in this area is rather superficial. Clearly more research is required to gather direct evidence for the involvement of NO in wound healing and to find out the mechanisms of action of NO on ECM metabolism.

Accumulating evidence indicates that TGF-β inhibits NO production by macrophages, epithelial and endothelial cells. It would be interesting to know if TGF-β also inhibits NOS expression and NO production by fibroblasts. Furthermore, the mechanisms of the inhibitory effect of TGF-β on NO production are worth of investigation. One approach would be to look for a TGF-β response element in the promoter regions of NOS genes.

It is intriguing that NO/proxynitrite stimulates the production and activation of metalloproteinases. This finding was based on the observation that IL-1, TNF-α, and IFN-γ treatment increases the NO and collagenase production by mesangial cells and chondrocytes *in vitro* (Murrell et al, 1995; Trachtman et al, 1996). The collagenase production and activation can be blocked by NOS inhibitor or NO scavengers such as hemoglobin. It is known that these proinflammatory cytokines can induce collagenase expression. However, the mechanisms of action are not clear. It would be interesting to know whether these effects are direct or indirect, through the NO signal pathway.

The data in this thesis showed a reverse correlation between TGF-β and NO in HSc fibroblasts, i. e. HSc fibroblasts express more TGF-β while producing less NO. However, it is not known if there is a cause-effect relationship between them. Resolving the mystery of the relationship between these two molecules may be helpful in preventing HSc or in designing therapeutic approaches for this disorder.

It is of interest that IFNs have opposite effects on TGF-β synthesis and NO production by fibroblasts. IFNs are well-known to have anti-fibrogenic effects. However, the mechanisms of the anti-fibrogenic effects of IFN are not known at present. This research provided indirect evidence indicating that IFNs might exert their anti-fibrogenic effects by inhibiting TGF-β expression and stimulating NO production by fibroblasts. More studies should be useful to gather more direct evidence for the mechanisms of action for IFNs on fibroproliferative disorders.

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